DIFFERENT ASPECTS OF THE BIOLOGY OF PHLEBOTOMINE SAND FLIES (DIPTERA: PSYCHODIDAE) AND THEIR AGE STRUCTURE IN A FOCUS OF CUTANEOUS LEISHMANIASIS IN NORTHEASTERN COLOMBIA

By

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I dedicate my dissertation to my parents who always encouraged my search for knowledge.

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Abstract of Dissertation Presented to the Graduate School
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DIFFERENT ASPECTS OF THE BIOLOGY OF PHLEBOTOMINE SAND FLIES (DIPTERA: PSYCHODIDAE) AND THEIR LONGEVITY IN A FOCUS OF CUTANEOUS LEISHMANIASIS IN NORTHEASTERN COLOMBIA

By

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Major Department: Entomology and Nematology

Different biological aspects of the reproduction of _Lu_. _anthophora_ were investigated. Longevity of different _Lutzomyia_ species from a focus of leishmaniasis in the Departmento of Norte de Santander, Colombia, was determined.

Maximum number of 1-day-old females was inseminated by 4-day-old males. Males became sexually mature 13 to 24 hrs after emergence and two-day-old males inseminated the maximum number of females. Male reproductive organs became smaller due to aging and multiple matings. The males accessory gland contained 2 types of secretions.

Trypsin- and chymotrypsin-like enzymes were quantitatively determined in immature and adult sand flies. More chymotrypsin-like enzymes were present in the larvae, newly emerged, sugar fed and females 72 hrs after blood feeding. Blood fed females had more trypsin-like enzymes. Twelve, 8, 4, 4, 5 and 11 different isozymes were secreted by larvae, early pupae, late pupae, newly emerged females, sugar fed females and blood fed females respectively. Eight, 1, 1 and 4 isozymes were trypsin-like in larvae, early pupae, late pupae and blood fed females respectively and the rest were chymotrypsin-like isozymes.

Biosynthesis, of JH III by the corpora allata _in vitro_ was analyzed by high pressure liquid chromatography and gas chromatography. Peak JH III biosynthesis occurred 24 hrs after emergence. The rate of JH III biosynthesis declined in 3-day-old sugar fed females and immediately following blood feeding. The biosynthesis however, peaked at 4 hrs. A rapid decline occurred after 4 hrs and the minimum rate of biosynthesis was found 30 hrs after blood feeding. The rate of JH III biosynthesis increased at 38 hrs after blood feeding and the maximum biosynthesis was after 96 hrs.

The females were capable of _leishmania_ transmission throughout the year and completed 1-4 gonotrophic cycles. _Lu_. _spinicrassa_, a probable vector of _Le_. _brazilensis_ completed 2 gonotrophic cycles from May-December.

CHAPTER 1
INTRODUCTION

Medical Importance of Sand flies

Sand flies are the sole vectors of *Leishmania*, a group of parasites that causes a number of enzootic and zoonotic diseases collectively referred to as leishmaniasis. According to a report of WHO (1984) this protozoan pathogen annually torments more than 400,000 people worldwide. Human leishmaniasis is caused by at least 14 different species of the genus *Leishmania*. Sand flies are widely distributed and their ranges extend from approximately 50° N to 40° S. In the Old World, leishmaniasis is transmitted by sand flies belonging to the genus *Phlebotomus* Rodani and Bert. It's distribution is mostly temperate and encompasses areas such as Central Asia, India, North Africa, and Mediterranean Europe. In the New World, leishmaniasis is transmitted by sand flies of the genus *Lutzomyia* Franca and its distribution is mostly tropical except for few autochthonous cases that were recorded in Texas (Lawyer 1984).

Sand flies are also vectors of other diseases. In the New World, a bacterium, *Bartonella* *bacilliformis* is commonly referred to as Carrion's disease that has two clinical forms, Oroya fever and Verruga peruana. The disease has a limited distribution, having been recorded from Peru, Ecuador, and Colombia. Lutzomyia verrucarum (Townsend) is the probable vector (Hertig 1939).

Arboviruses belonging to three families (Rhabdoviridae, Reoviridae, and Bunyaviridae) are transmitted by sand flies (Tesh et al. 1971b; Tesh et al. 1987; Tesh 1988; Tesh et al. 1989). Transovarian transmission of Phleboviruses has been shown for three Lutzomyia species (Endris et al. 1983; Jennings & Boorman 1980; Tesh & Modi 1983). Viruses of seven New World serotypes were isolated from male sand flies (Tesh 1988), and Phlebovirus was also isolated from both sexes of sand flies in Panama (Tesh et al. 1974). In the Old World, viruses belonging to the above three families have been isolated from Phlebotomus sand flies. The best known disease in Old World is papatacci fever, or sand fly fever, or 3-day fever reported from India, China, Central Asia, and the Mediterranean region.

American Leishmaniases

A large number of Leishmania species coexist in the Americas, and together with these a number of morphologically similar nonleishmanial trypanosomatids parasites occur in many localities (Young & Lawyer 1987). This coexistence complicates the studies of vector implication of different sand flies in an area. The situation is further complicated due to the presence of certain host specific mammalian leishmaniasis species (*Le. hertigi* of porcupines) that have never been reported to infect humans (Young & Lawyer 1987). The occurrence of sympatric morphospecies of *Lutzomyia* in certain foci of leishmaniasis further complicates the situation (Young & Lawyer 1987).

In the New World two clinical forms of leishmaniasis, visceral and cutaneous are often recognized, but the sympotoms alone do not indicate the identity of the parasite. In certain types of visceral leishmaniasis, dermal lesions have been observed. The genus *Leishmania* is divided into two sub-genera, *Leishmania* and *Viannia* according to their mode of growth and other parameters. The subgenera correspond to two sections, Peripylaria and Suprapylaria (Young & Lawyer 1987). The section Peripylaria contains *Le. braziliensis braziliensis*, *Le. b. guyanensis*, *Le. b. panamensis*, *Le. b. peruviana* and species of *Leishmania* from armadillos. Section Suprapylaria contains *Le. donovani chagasi*, *Le. hertigi hertigi*, and *Le. h. deanei* (from tree porcupines in the neotropics). Also included are *Le. mexicana mexicana*, *Le. m. aristedesi* (from rodents), *Le. m. enreittii* (From domestic guinea pigs), *Le. m. garnhami*, *Le. m. venezulensis*, *Le. m.* subspecies from Matto Grosso State, Brazil, *Le. m.* subspecies from Minas Gerais State, Brazil, and different *Leishmania* subspecies from the

Dominican Republic. The distribution and vectors of all these species were reviewed by Young and Lawyer (1987).

Effects of Leishmania Infection on Sand Flies

Recent improvements in the rearing techniques of sand flies (Young et al. 1981; Endris et al. 1982) have enabled many scientists to experimentally infect sand flies with different species of *Leishmania* and study their course of development. Sand flies are pool feeders and their mouth parts and digestive system are described in chapter 2. Sand flies that feed on the periphery of the leishmaniasis lesion ingest the parasite in a form that is called an amastigote. Since sand flies are pool feeders, they can ingest only a few amastigotes with their blood meal. These amastigotes are then released due to the rupture of haemocytes during feeding perhaps by the cibarial teeth (Lewis 1975). The number of amastigotes ingested by sand flies partly depends on the length of their mouth parts (Young & Lawyer 1987). Sand flies inflict injury to the host by lacerating the tissues. This damage may attract macrophages in the vicinity of the feeding site, thus increasing the chances for the parasite to reach its site of extrinsic development in the sand fly midgut.

Reptilian *Leishmania* and *Trypanosoma* are capable of thriving in their sand fly vectors even when they are ingested with nucleated reptile blood (Adler 1964).

Although human and mammalian blood causes mortality in *Leishmania* cultures, this effect is immediately removed after the ingestion of the parasite by the sand flies (Adler & Theodor 1930). Human serum can inhibit the rate of trypsin production in the midgut of sand flies. Low rate of blood digestion is favorable for the growth of *Leishmania* (Borovsky & Schlein 1987).

After reaching the sand fly midgut, the amastigotes are released from the macrophages and are now present inside the peritrophic membrane where they undergo either one or two divisions and transform into flagellate forms, promastigotes. The rate of formation of the peritrophic membrane may be directly involved in the susceptibility or refractoriness of sand flies to various parasites because leishmanial promastigotes cannot penetrate it (Killick-Kendrick 1979).

The Old World species of *Leishmania* can be transmitted only by their particular host sand flies. In *Lu*. *abonnenci* (Floch & Chassignet) females, fed on mice infected with *L*. *mexicana* *mexicana*, the parasites were embedded in the peritrophic membrane as early as 18 hrs after a blood meal (Walters et al. 1987). The first division of the parasite occurs within the first 24 hrs after the ingestion of an infective blood meal (Strangeway Dixon & Lainson 1966). These actively dividing parasites lie within the bag of the peritrophic membrane (Leany 1977). Thus, it may be possible that rate of formation of the peritrophic membrane varies in different species of sand flies. In some species it acts as a barrier to massive transfer and establishment of the promastigotes in the microvilli of the midgut epithelial cells.

The nature of the peritrophic membranes of sand flies also affects their susceptibility to *Leishmania*. Feng (1951) studied the peritrophic membrane in *Ph*. *chinensis* (Newst), *Ph*. *mongolensis* (Sinton), and *Sergentomyia squamirostris* (Newstead). He postulated that in poor hosts of *Leishmania* such as *Ph*. *mongolensis*, the peritrophic membrane remained intact and the parasites passed out in feces within it. On the other hand, in susceptible species such as *Ph*. *chinensis* the peritrophic membrane was broken three days after a blood meal. Thus, leishmanial promastigotes were able to escape and establish an infection in the anterior midgut. The transformation of amastigotes to promastigotes takes 2-3 days (Molyneux & Killick-Kendrick 1987). The promastigotes develop flagellar expansion or hemidesmosomes that help to attach them to either microvilli of midgut or cuticle of the pylorus or ileum and transform into paramastigotes. Paramastigotes undergo an extensive multiplication before migrating to the esophagus and pharynx and transform into promastigotes. Later the promastigotes migrate to the proboscis.

Free floating flagellates of Leishmania mexicana were observed in the lumen of the Malpighian tubules and the pylorus of field collected Lutzomyia olmeca (Fairchild & Theodor)(Williams 1970) and also in many laboratory infected sand flies (Walters et al. 1987; Christensen & Herrer 1980). The Malpighian tubules may serve as sites providing nutrition to the parasites after the complete digestion of the blood meal (Christensen & Herrer 1980; Lawyer 1984; Walters et al. 1987).

At the completion of the extrinsic development of the Leishmania parasites, small actively moving forms are found within the mouth parts and the different parts of the midgut. These small forms may attach to the chitin or may occur free in the pharynx or in the mouth parts of the sand flies (Killick-Kendrick 1979). The attachment of the parasites in the mouth parts results in impaired function of the cibarial receptors (Killick-Kendrick et al. 1977b). Infected Lu. longipalpis females take a very small subsequent blood meal or sometimes are unable to feed at all (Adler & Bur 1941). Such flies transmitted maximum number of L. mexicana amazonensis infections (Killick-Kendrick et al. 1977b).

The salivary gland lysate of Lu. longipalpis (Lutz & Neiva) possesses a property of enhancing the extent of cutaneous infection by Leishmania major in BALB/C mice, which are genetically susceptible to Leishmania infection and CBA mice which are genetically resistant to Leishmania infection. Injection of Leishmania major together with salivary lysate results in cutaneous lesions which are five to ten times as large and contain as much as 5,000 times more parasites than the controls. In inocula that contains smaller number of parasites, the parasites are detected in the lesion only when they are injected together with the salivary homogenates. The lysate is capable of producing an enhancing effect in quantities as small as 10% of the total gland homogenate (Titus & Riberio 1988).

Many methods of Leishmania transmission are suggested. The first mode of transmission is by the bite of an infected sand fly. The second method known as "blocked fly" theory suggests that in infected sand flies the leishmanial parasites infecting the stomodeal valve can be back washed into the feeding site by the blood meal. This happens due to the blockage of the normal blood flow in the posterior midgut after the congestion of the stomodeal valve by the parasites (Jefferies et al. 1986). The concept of blocked flies is further supported by the presence of gel-like matrix in the cardia and stomodeal valve regions of the infected Lu. abonnenci (Floch & Chassignet) and Lu. diabolica (Hall)(Lawyer 1984; Walters et al. 1987). Few enzymes are found in the saliva of hematophagous insects. Presence of salvia is necessary for the intake of a blood meal in pool feeders such as tsetse flies compared to capillary feeders like mosquitoes (Benjamini & Feingold 1970).

Sugar is an essential component of the diet of sand flies (Kirk & Lewis 1951; Lewis & Domoney 1966; Chaniotis 1974; Young et al. 1980). Its presence in the diet of sand flies enhances their chance of transmitting leishmaniasis to susceptible hosts (Smith et al. 1940, 1941; Swaminath et al. 1942; Short 1945; Killick-Kendrick et al. 1977b; Young et al. 1980). Presence of sugars in the crop of infected flies also enhance the rate of development of Leishmania (Killick-Kendrick 1978).

These sugars also act as food for the leishmanial parasites present in the foregut, which is lined with relatively impermeable cuticle (Davis 1967). Motile flagellates of Leishmania mexicana mexicana were occasionally seen in the crop of Lutzomyia abonnenci (Walters et al. 1987). Similarly, Hertig and McConnell (1963) also reported Leishmania promastigotes in the crops of Lutzomyia sanguinaria (Fairchild & Hertig).

Life Cycle of Phlebotomine Sand Flies

A newly emerged female usually seeks a blood meal 12-24 hrs after emergence. Males usually emerge earlier than females and are ready to mate after one day. Mating of sand flies may take place at the breeding site or on or near a host (Chaniotis 1967). Males usually outnumber females at the resting sites (Alexander 1988). The male to female ratio observed in the colony cages is mostly 1:1; perhaps in nature females move to blood feeding sites shortly after mating. Females can complete 1 to 4 gonotrophic cycles in the laboratory (Ward 1977; Chaniotis 1986) and also in nature (see chapter 6). The number of eggs that develop during one gonotrophic cycle depends upon the protein content of the blood meal (Ready 1979). In laboratory colonies of Lu. trapidoi (Fairchild & Hertig), survival of females increased in the presence of high male to female ratios during oviposition and females completed upto 3 gonotrophic cycles (Chaniotis 1986). Females that laid eggs within 4 days after blood feeding laid large number of eggs and survived longer than those that laid 9 days after blood feeding (Chaniotis 1986).

Females that take a sugar meal before blood feeding develop more eggs than those that are starved before a blood meal (Ward 1977). In nature some species are autogenous and lay their first batch of eggs without blood feeding (Johnson 1961).

Phlebotomine sand flies deposit their eggs in the detritus and the eggs hatch after about 8 days in non-diapausing species. Larvae feed on organic detritus, dead leaves, and fungi (Johnson & Hertig 1961). The first instar larvae are whitish in color, lack legs, and possess a pair of caudal setae. The first instar larvae molt after 5 or more days to second instar. The second instar molts to third instar, fourth instar and finally to pupae. The whole growth takes from 15-30 days.

Sand flies diapause in the larval stage and it is often due to changes in day length and temperature. In New World species diapause in larvae is induced due to insufficient humidity at the start of the dry season or may occur in the egg stage (Hanson 1968). Pupae eclose to adult sand flies after 8 or more days depending on temperature and sand fly species. No information is available about the occurrence of circadian rhythms during adult emergence.

Objectives

The present investigation was carried out to obtain information about some important aspects of sand fly biology. These includes the following studies:

1) The mating behavior of males and females and the effects of age and matings on the morphology of male reproductive system.

2) Production of trypsin- and chymotrypsin-like isozymes was investigated in both immature and adult Lu. anthophora. Poor information is available about Trypsin- and chymotrypsin-like isozymes of adult sand flies (Schlein et al. 1983; Schlein & Romano 1986; Borovsky & Schlein 1987) and no information is available about the enzymes in immature sand flies.

3) Juvenile hormone III (JH III) production was investigated in female Lu. anthophora. Previous studies on mosquito physiology has shown that some of the most important aspects of an insect's life, such as host seeking behavior, mating, and vitellogenesis are controlled by juvenile hormones (Meola & Petralia 1980; Borovsky 1984; Meola & Readio 1987). The recent advances in tissue culture techniques of corpora allata (Borovsky unpublished) greatly facilitated the in vitro production of JH by the corpora allata of unfed, sugar fed and blood fed female Lu. anthophora.

4) All the above topics are important features of egg development. Thus, egg development was studied in Lutzomyia anthophora (Addis). Finally, the most important aspect that results from the above processes is oviposition and survival of sand flies in nature. Eight different Lutzomyia species, collected during 1985-1988 from two leishmaniasis foci, Arboledas and Durania from Norte de Santander in Colombia, were age graded according to the methods of Detinova (1962). Their longevity in relation to Leishmania transmission is discussed.

CHAPTER 2
LITERATURE REVIEW

Reproductive Biology of Sand Flies

Reproduction, the most important aspect of an insects's life, is an intricate process. It encompasses many other aspects such as larval nutrition, environmental factors, mating, host finding, feeding, egg development and oviposition. Each one of the above processes is an entity in itself and is comprised of a network of behavioral and physiological events.

Mating consist of behaviors like courtship or epigamic behaviors that bring the two sexes together and results in the insemination of the female. After mating, host finding behavior is invoked. In some mosquito species, release of juvenile hormone (JH) is the trigger for host seeking behavior (Meola & Petralia 1980; Meola & Readio 1988), whereas in others, JH does not seem to play a part in host seeking behavior (Lea 1963; Bowen & Davis 1989).

Certain sand fly species develop their first clutch of eggs without a blood meal (autogenous), whereas others require a blood meal (anautogenous) for the completion of first and later gonotrophic cycles. Rate of blood digestion depends on factors such as environmental temperatures (Weitz & Buxton 1953; Yang & Davis 1968; Honda et al. 1985), presence or absence of parasites in the blood meal (Borovsky & Schlein 1987), and mating (Edman 1970; Downe 1975; Adlakha & Pillai 1976a; Houseman & Downe 1986).

In female mosquitoes and other haematophagous insects, different consequences of mating, alteration of blood meal size (Adlakha & Pillai 1976a), stimulus for oviposition (Leahy & Craig 1965), and initiation of a refractory period to further mating (Young & Downe 1982, 1983) have been observed. Blood proteins are utilized for the development and maturation of eggs. Presence of JH makes the ovaries receptive to egg development neurosecretory hormone (EDNH) and induces responsiveness in the fat bodies to 20-hydroxyecdysone (Flanagan & Hagedron 1977; Shapiro & Hagedron 1982).

The reproductive biology of haematophagous insects represents a network of processes that begins from larval nutrition and exposure to different environmental conditions and ends after oviposition. In autogenous species of mosquitoes the number of eggs laid by a female is directly dependent on the larval nutrition, whereas in anautogenous species it also depends on the size of the blood meal and the species of the host. Many autogenous species of mosquitoes and sand flies require a blood meal for egg development during the second gonotrophic cycle.

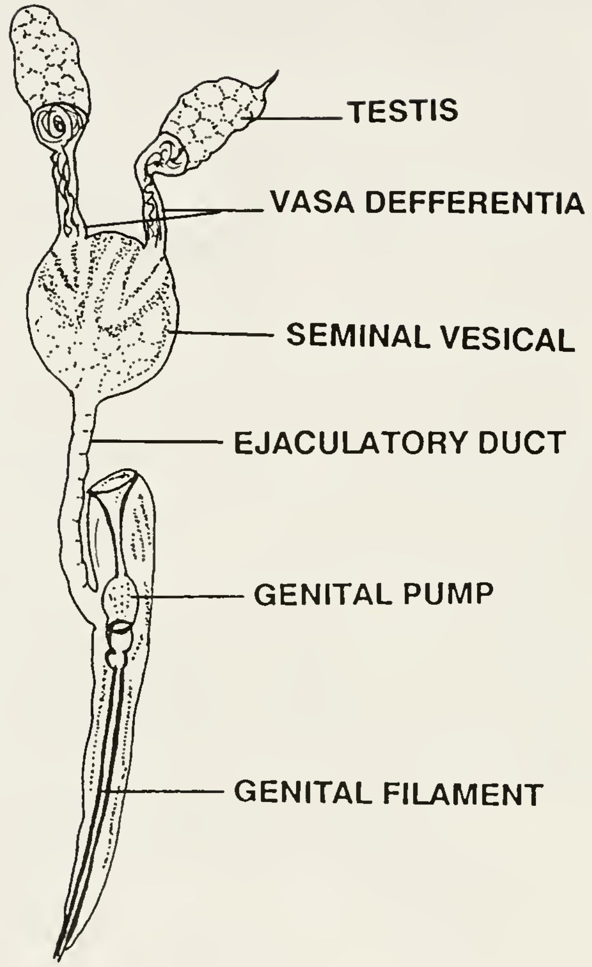

Figure 2-1. Male reproductive system of Lutzomyia sauroida

Morphology of the Male Reproductive System

The male reproductive system consists of a pair of rounded, oblong, or oval testes, a large pear-shaped, conical or almost spherical vesicula seminalis, the sperm pump, and the sperm tubes (Perfil'ev 1968; Sherlock & Carneiro 1964). The vasa efferens vary in size in different species of sand flies and open into the vesicula seminalis by pressing part of its wall inward (Fig. 2-1). The vesicula seminalis opens into a narrow ejaculatory duct which leads to the genital pump (sperm pump). The genital pump is a syringe-shaped organ having two long sperm tubes or filaments arising from it (Sherlock & Carnerio 1964; Downes 1968). The filaments lie in the grooves of the aedeagus where they can move freely. The filament emerges from the apex of the aedeagus, or slightly proximal to it depending on the shape of the aedeagus.

In *Phlebotomus papatasi* (Scopoli) the filaments are short in length and are only inserted into the tip of the spermathecal orifices. In other species the filaments are longer and are inserted well inside the spermathecal ducts, sometimes reaching into the spermathecae (Sinton 1925). In species having common spermathecal opening, the filaments remain paired; e.g., in *Phlebotomus melloni* (Sinton) the filaments or the aedeagal ducts remain paired and are inserted together in the spermathecal duct and at the apex the filaments are inserted into the separate spermathecae (Sinton 1932).

Sherlock and Carnerio (1964) described the male reproductive system of different sand flies and emphasized that the differences observed in fresh specimens could be used to identify the different species. They described the male reproductive system as having two testes, two efferent tubules, one seminal vesicle, one deferent tubule (= ejaculatory duct of Perfil'ev 1968), one ejaculatory pump (= genitial pump of other authors). They noted that spermatozoa were cane shaped. During mating spermatozoa passed through the seminal vesicles and were deposited together with the seminal liquid into the female spermathecae. In males which had not mated the spermatozoa were in the form of balls in each testis.

Dallai et al. (1984) studied the evolution of sperm in the Phlebotominae and their relation to other Nematoceran Diptera. The spermatozoa of Ph. papatasi (Scopoli) are thread like, 13 µm long and 0.5 µm wide, head is 2.5 µm long with a compact electron-dense fusiform nucleus and chromatin is absent in its anterior end. The mature spermatozoon has a longitudinally arranged distal centriole that is continued into a 10 µm long axoneme. The axoneme has a "9 + 9 + 0" arrangement of the microtubules. The axonemes are motile inside the spermathecae. The structure of the tail part is similar to other insects.

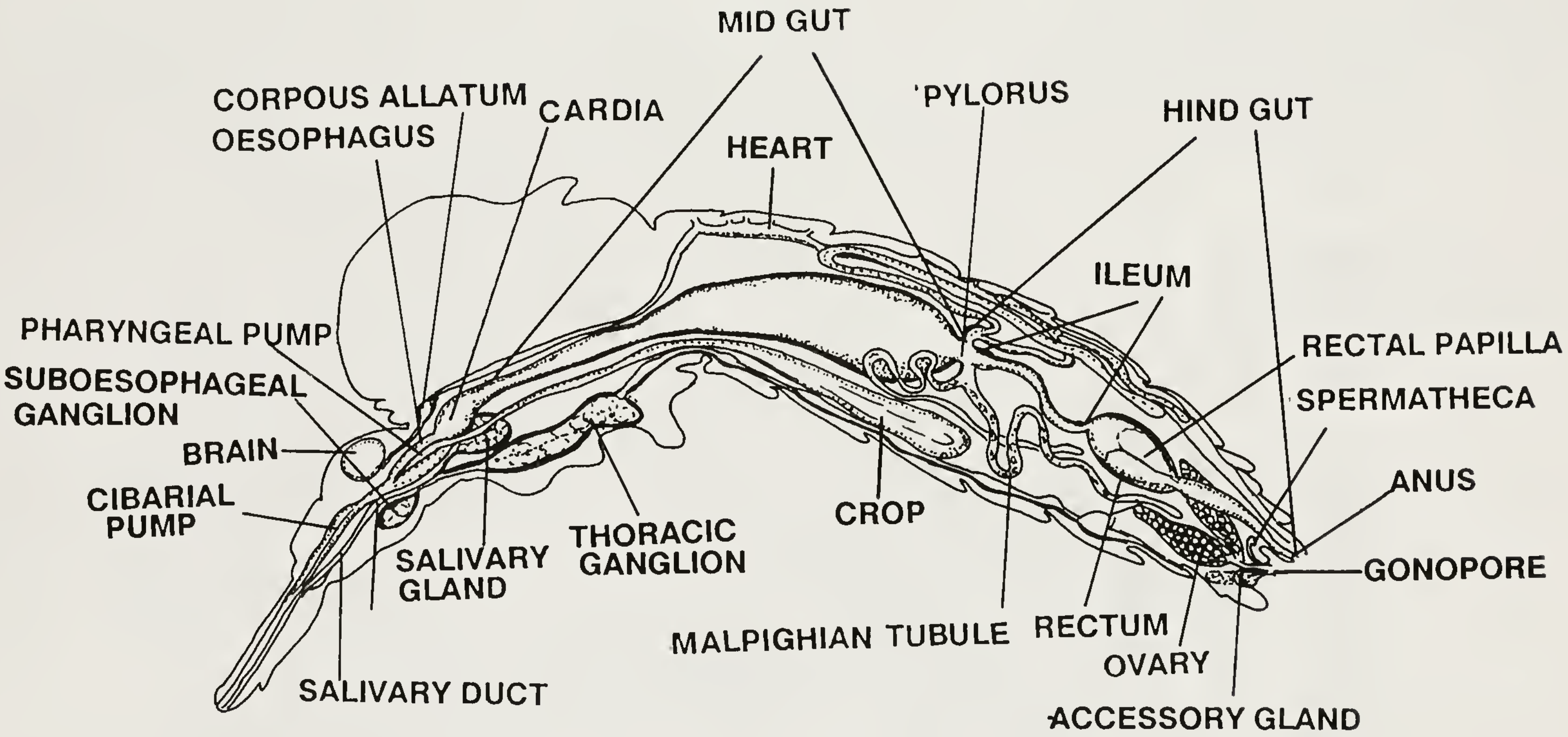

Figure 2-2. Internal anatomy of Lutzomyia sand flies showing various organs.

Digestion of Food by Sand Flies

Digestive System of Phlebotomine Sand flies

Mouth parts. Sand flies have a piercing sucking fascicle or syntrophium (Jobling 1976), and an archaic type proboscis (Lewis 1974). The proboscis extends into the buccal cavity or cibarium (Fig. 2-2). Variable number of horizontal, vertical, and lateral teeth are present in the cibarium of *Lutzomyia* species. From the ventral aspect, the horizontal or hind teeth have either pointed or blunt tips (Young 1979). The vertical teeth are distal to the horizontal teeth and sometimes appear as dark dots. The lateral teeth occur next to horizontal teeth on both sides of the cibarium. Which connects posteriorly to the pharynx and lies within the clypeus (Young 1979).

Lutzomyia sand flies that feed on mammals have long labra with hooked teeth. These teeth are also present on the paired maxillae. In comparison, most species that feed on reptiles have ridged tipped maxillae (Lewis 1975). Two types of sensillia are present on the mouth parts of sand flies. The first type occurs on the proboscis; the second type is on the cibarium (Killick-Kendrick & Molyneux 1981; Lewis 1984).

An aggregation pheromone occurs in the maxillary palps of *Ph. papatasi*. The response of sand flies to this

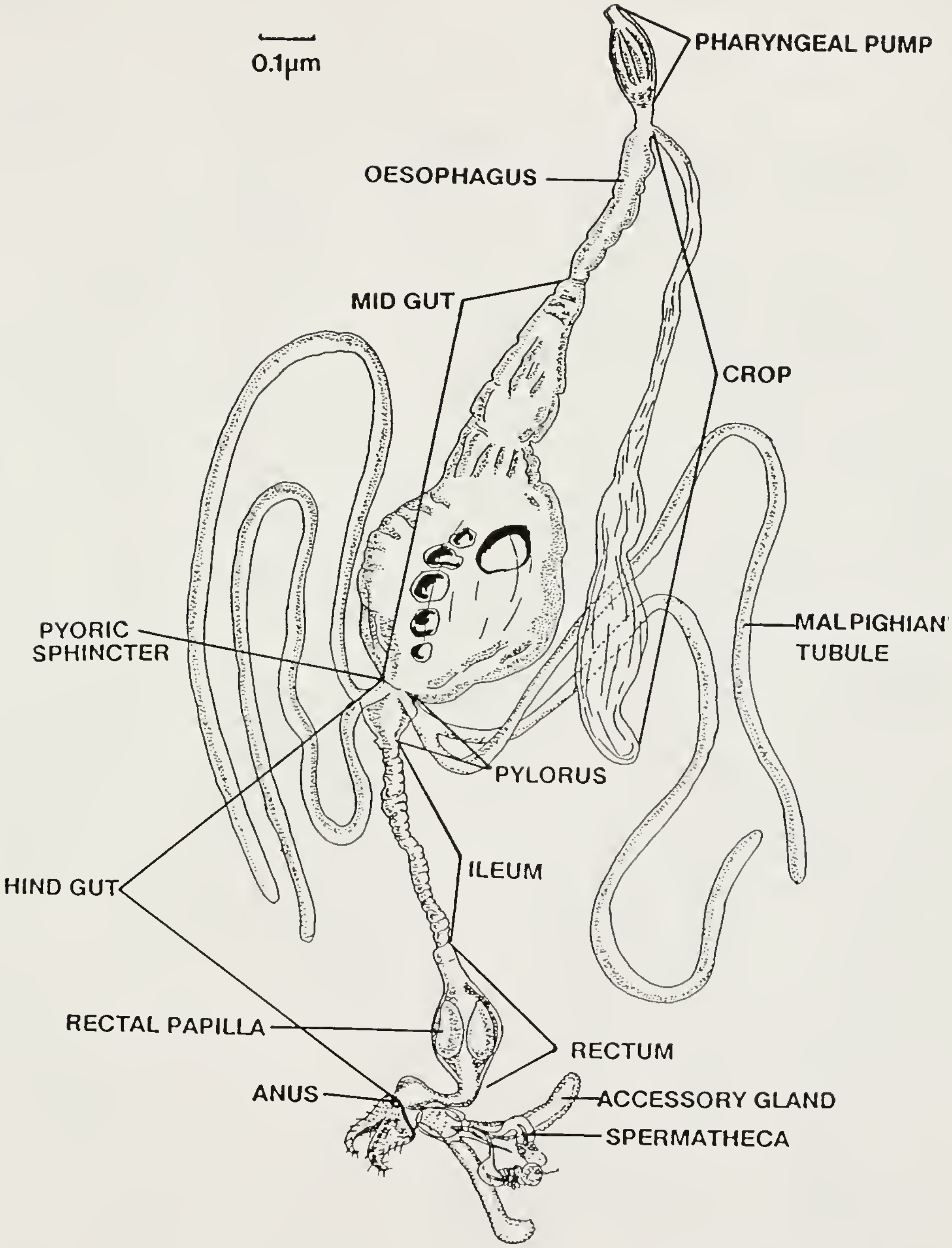

Figure 2-3. Digestive system of Lu. anthophora.

pheromone is olfactory and temperature dependent, suggesting it is volatile in nature (Schlein et al. 1984).

Sand flies have a pair of salivary glands situated at the junction of the head to the thorax (Jobling 1987; Lewis 1974) (Fig. 2-2).

Foregut. The alimentary tract of sand flies has three major regions, foregut, midgut, and hindgut (Fig. 2-2). The foregut consists of a proboscis, cibarium, pharynx, crop, oesophagus, and stomodeal valve. The pharynx is divided into an anterior and a posterior pharynx. The latter has an armature that is composed of three chitinous plates. Most Lutzomyia species have transverse unarmed ridges on this region, and in few species it is armed with spines (Young 1979). The pharynx opens into the oesophagus, which is connected via a stomodeal valve to a small wide slightly bulbous portion, the cardia or cardiac stomach (Gemetchu 1974; Walters et al. 1987) (Fig. 2-3). The stomodeal valve in Phlebotomus longipes (Parr) is formed by the protrusion of the oesophagus as a thick ring of elongated cells into the lumen of the cardia. On the hemocoelic aspect, this ring is covered with fine muscle strands.

Crop. The crop is ballon shaped and opens at the junction of the oesophagus to the cardia (Figs. 2-2 & 2-3). Sugar solutions imbibed by the sand flies go into the crop where they play an important role in maintaining energy for the growth of Leishmania parasites.

Midgut. The midgut is divided into an anterior and a posterior midgut. The cardia is the first part of the anterior midgut and is continuous with the thoracic portion of the anterior midgut. Both the cardia and anterior midgut are found between thoracic muscles (Jobling 1987; Lewis 1974; Walters et al. 1987) (Figs. 2-2 & 2-3). Some authors consider the whole anterior midgut lying in the thoracic muscles as the cardia (Adler & Theodor 1926; Hertig & McConnell 1963), whereas, Davis (1967) considers the slightly bulbous area following oesophageal-crop junction as the cardia (Fig. 2-3). The cells of the anterior midgut are cuboid, with striated borders or microvilli (Gemetchu 1974). The anterior midgut is continuous with the posterior midgut.

Ultrastructure of the posterior midgut was described for two species of sand flies (Gemetchu 1974; Rudin & Hecker 1982). Ultrastructure of the posterior midgut of *Lu. longipalpis* (Lutz & Nieva) shows distinct differences between sugar fed and blood fed females (Rudin & Hecker 1982). The midgut of sugar fed female shows a single layered epithelium of highly polarized elongated cells of relatively small size compared to cells of blood fed females. An oval nucleus, 10% of the cell volume, occurs in the center of the cell. Triglyceride-like structures are found around the nuclear region in association with the myelinated bodies and lysosome like structures. About 15% of the cell cytoplasmic volume contains randomly distributed mitochondria. Free ribosomes are present throughout the cells. Smooth endoplasmic reticulum occurs in association with the golgi bodies that are regularly distributed throughout the cells. Various amount of vesicles, cisternae, and whorls of rough endoplasmic reticulum are present in the cells and accounts for 5.5 m^2/ m^3 of the cytoplasm.

The epithelial cells show densely packed microvilli, which are about 1 mm long and make up to 8% of the cytoplasmic volume in Lu. longipalpis. The cells also show a cytoskeleton composed of many microtubules and microfilaments. Cells of other haematophagous insects also have a large amount of endoplasmic reticulum and enzyme synthesis may occur there. The endoplasmic reticulum involved in enzyme synthesis is secluded from the rest of the cell cytoplasm to prevent it from auto-digestion (Richards 1975).

A thin basal lamina separates the stomach epithellium of Lu. longipalpis from the hemocoel. Gaps and septate junction are present between the lateral cell walls. Only few regenerative cells are present. The midgut is surrounded with longitudinal and circular muscles (Rudin & Hecker 1982). The inner surface of the midgut is devoid of cuticle but this does not mean that inner midgut epithelium is directly exposed to the blood meal. An acellulary secreted peritrophic membrane is present between the midgut epithelium and the blood meal.

A brush border formed of microvilli is present in both the anterior and posterior midgut portions of Ph. longipes and Ph. sergenti (Parr)(Perfil'ev 1928; Gemetchu 1974), while it is present only in the anterior midguts of Ph. orientalis (Pass) and Ph. papatasi (reference not seen, but referred in Gemetchu 1974).

In unfed insects, the midgut cells are inactive and the ribosomes are localized on balls, whorls or sheets of rough endoplasmic reticulum (Gemetchu 1974; Richards 1975). The midgut epithelium of Ph. longipes and Lu. longipalpis becomes flattened and the whorls of the endoplasmic reticulum open after the ingestion of blood meal. Mean volume of microvilli, rough endoplasmic reticulum, and smooth endoplasmic reticulum of the midgut epithelial cells of Lu. longipalpis increases within 24 hrs after the ingestion of a blood meal. Some of the parameters described above decrease 2 days after ingestion of a blood meal (Rudin & Hecker 1982). Under different physiological conditions, similar changes occur in the midgut structure of Ph. longipes (Gemetchu 1974).

Electron microscopical studies of the midgut of mosquito Aedes aegypti (L) show that it has three types of cells, a. digestive cells (Hecker et al. 1971), b. endocrine cells (Brown et al. 1985) and c. regenerative cells (Hecker et al. 1971).

The midguts of mosquitoes show changes similar to those observed in sand flies before and after the ingestion of a blood meal. In unfed female _Ae_. _aegypti_ the digestive cells of the midgut epithelium contain no secretory granules (Hecker et al. 1971). In blood fed mosquitoes, the surface of the rough endoplasmic reticulum greatly increases by the opening of its tight whorls, a condition existing in sugar fed and/or starved mosquitoes (Hecker et al. 1971; Hecker & Brun 1975; Bauer et al. 1977). The secretory granules appear in increasing number 8 and 24 hrs after a blood meal (Rudin & Hecker 1979).

The above observations are also supported by a recent report in which free and membrane-bound ribosomes of intestinal cells of female _Ae_. _aegypti_ were quantified during the digestive cycle (Gander et al. 1980). Free ribosomes increase up to 24 hrs after blood feeding, reach a plateau and decline to the unfed level after 96 hrs. Similarly, membrane-bound ribosomes increase up to 36 hrs and decline to the unfed level after 96 hrs (Gander et al. 1980). Increase in the amount of rough endoplasmic reticulum after a blood meal is thought to be due to the production of trypsin-like enzymes (Rudin & Hecker 1979). Contrary to previous reports, in mosquito _Anopheles_ stephensi (Liston) secretory granules were found before the intake of a blood meal (Berner et al. 1983).

In Ph. longipes some increase in the vacuolation of the cytoplasm and in the labyrinth spaces of the basal membrane occurs after the ingestion of a blood meal. This increase is probably due to the transport of digestion products across the epithelial cells and through the basal membrane to the haemocoel (Gemetchu 1974).

Peritrophic membrane. Hematophagous insects, except fleas, synthesize a peritrophic membrane after ingesting a blood meal (Richards & Richards 1971).

Peritrophic membrane is absent in unfed and sugar fed female sand flies (Gemetchu 1974). After blood feeding development of the peritrophic membrane is initiated within 30 minutes after ingestion of a blood. A secretion, present between the microvilli within 12 hrs post feed, extends to the microvilli tips and reaches into the lumen of the midgut. A thin sac of this secretion is present around the blood bolus within 24 hrs post feed. This sac gradually thickens and becomes stronger. After 48 hrs the peritrophic membrane can be dissected away from the midgut epithelium. It is present mainly in the midgut but a small extension continues in the posterior end of the foregut and this portion is much folded (Gemetchu 1974).

Two and three days after ingestion of a blood meal, the digested blood, now dark brown, is present in the form of a rod in the anterior part of the peritrophic membrane. Similar rod shaped particles are also present inside the blood meal and in the ectoperitrophic space.

The ectoperitrophic space occurs outside the peritrophic membrane and inside the midgut epithelium. The peritrophic membrane starts to break down at its anterior end at about the third day. It breaks around the blood meal by the fourth and fifth day. Sand flies defecate the undigested blood and remaining peritrophic membrane on the sixth or seventh day after blood feeding (Gemetchu 1974).

If a partially blood fed Ph. longipes refeeds, the second blood meal envelopes the first peritrophic membrane containing the first blood meal. This results in a slower rate of disintegration of the first peritrophic membrane. The rate of digestion of the first blood meal is also slower and may be due to its separation from the midgut epithelium by the second blood meal (Gemetchu 1974).

The electron microscopy of the peritrophic membrane just after engorgement reveals an irregular lattice like structure and a network of fibrils. The peritrophic membrane, rich in proteins and some of the hexoses, is insoluble in water, alcohol and KOH (Gemetchu 1974).

The process of peritrophic membrane secretion may involve the interaction of proteolytic secretions at their interface with the blood mass. This process may become weak as blood digestion proceeds to its completion (Gemetchu 1974).

Thickness of the peritrophic membrane varies in different species of insects and also in different instars of the same species. It may be a granular sheath or it may have layers of fibrous material under the granular layer. The microfibrous layer may be arranged randomly or in order of hexagonal or orthogonal arrays (Peters 1976).

Insects ingest their food in macromolecular form, such as polysaccharides, proteins, and lipids (triglycerides, phospholipids and glycolipids). These large molecules can not pass the peritrophic membrane and midgut epithelium; therefore, they are broken down in to small peptides, amino acids by trypsin-like enzymes. These enzymes are reported from many larvae and adults of insects, such as stable fly Stomoxys calcitrans (L), house fly Musca domestica(L), common blow fly Calliphora vomitoria (L), mosquito Ae. aegypti, and Culex nigripalpus (Theobald) (Champlain & Fisk 1956; Yang & Davis 1971; Borovsky 1985; Patterson & Fisk 1958; Greenberg & Parestky 1955; Patel & Richards 1960; Fraser et al. 1961; Gooding 1966a, 1966b; Borovsky 1986). There is direct evidence that midgut epithelium cells of mosquitoes produce these proteases (Briegal & Graf 1989). A high level of proteolytic activity, with specificity to the trypsin- and chymotrypsin-like substrates, was also detected in the midgut of detritus-feeding larvae of the crane fly

Tipula abdominalis (Say) (Sharma et al. 1984). Who suggested that these enzymes were secreted from the midgut epithelium cells and not from the microbes originating from the detrital food.

The peritrophic membrane is selectively permeable to small peptides, amino acids, water, enzyme amylase, and poly peptide of up to 23,000 dalton molecular weight (Borovsky 1986). Borovsky (1986) proposed a system for blood digestion in mosquitoes, and wrote the following:

> A signal is sent to the midgut epithelial cells to initiate trypsin synthesis, and trypsin is secreted into the ectoperitrophic space. The trypsin traverses the pores in the peritrophic membrane and begins to digest the blood clot around its periphery, minimizing exposure to trypsin-inhibitory subfractions. Peptides, amino acids, and polypeptides, of molecular weight smaller than 23,000 daltons, diffuse out through the peritrophic membrane into the ectoperitrophic space. Poly peptides and peptides that has accumulated in the ectoperitrophic space are further digested into free amino acids, which are transported through the midgut epithelial cells into the hemolymph. The free amino acids are then used by the fat bodies to synthesize vitellogenin (egg yolk proteins). pp. 158-159.

The above theory is also supported by studies on Ae. aegypti that shows 60% of total proteolytic activity occurs around the peritrophic area, and the rest of the activity is inside the midgut epithelium and inner to the peritrophic membrane (Graf & Briegal 1982). This theory is further supported by a study in which polycolonal antibodies were developed against trypsin from the midgut of blood fed Ae. aegypti. These antibodies were employed to detect the trypsin activity in the midgut at different hours after a blood meal. Trypsin activity was detected in the lumen of the midgut 8 hrs after blood feeding. The trypsin activity was localized in the posterior distensible part of the midgut and was on the periphery of the peritrophic membrane and the blood bolus. Maximum trypsin activity was detected 24 hrs after a blood meal (Graf et al. 1986).

Secretory granules, labelled with anti-trypsin antibody and protein A-colloidal gold, were first detected 12 hrs after the blood meal, by electron microscopy (Graf et al. 1986). At 18 hrs post blood meal secretory pathway was traced from the cisternae of the golgi body where granules were formed to their release by exocytosis in the lumen of the midgut (Graf et al. 1986). Graf et al. (1986) also suggested that the changes in rough endoplasmic reticulum, after blood feeding, were not due to production of proteases as was suggested by many others (Frevogel & Staubli 1965; Hecker 1977; Berner et al. 1983), but were due to the formation of peritrophic membrane which is secreted in greater amount than the proteases.

Hindgut. The posterior midgut opens into the hindgut via the pyloric valve, which is similar to the stomodeal valve of Ph. longipes (Walters et al. 1987). Four Malpighian tubules arise at this junction (Figs. 2-2 & 2-3). Two Malpighian tubules arise together from a common origin on each side (Jobling 1987). The function of the Malpighian tubules is excretion of urine.

The hindgut is divided into the anterior intestine and the posterior intestine. The anterior intestine has three portions, i.e., pylorus, ileum, and colon (Figs. 2-2 & 2-3). A pyloric armature has been observed in the New World sand flies. The armature is spiculate and occupies more than posterior 1/3 of the pylorus. It is similar in structure to the pharyngeal armature of several Neotropical species and has posteriorly directed spines. It may function in the disruption of the un-digested hematin blood meal residue and its enclosing peritrophic membrane during the ultimate passage through the hindgut (Christensen et al. 1971).

The pylorus is connected to ileum, which is a narrow long tube (Figs. 2-2 & 2-3). The ileum of _Ph_. _papatasi_ is surrounded by circular muscles, which in turn are surrounded by longitudinal muscles. The whole ileum is enveloped on the outside with a peritoneal sheath (Jobling 1987). The ileum leads to the rectum, which has a pair of leaf like rectal papillae and opens to the outside through the anus.

The posterior intestine is subdivided into the rectal sac containing the rectal papillae and rectum. The inner surface of the hindgut, like the foregut, is lined with cuticle that is continuous with the cuticle on the outside of the animal. The cuticle of the foregut is relatively impermeable compared to that of the hindgut. Most of the absorption of nutrients occurs in the midgut. The rectum opens to the outside by an opening called the anus.

Different Enzymes Secreted during Digestion of Food

Enzymes are organic catalysts that enhance the rate of chemical reactions without changing themselves. The first array of digestive enzymes is found in the salivary glands of the blood-sucking insects. The next enzyme system occurs in the midgut of insects.

Salivary enzymes and their functions

Sand flies are pool feeders and have very small mouth parts, which can penetrate only the superficial layers of skin (Adler & Theodor 1926; Lewis 1975; Jobling 1987). They feed by lacerating the capillary loops in the superficial skin layers and ingest the blood pooling into the resulting hematomas (Riberio et al. 1986). The Old World sand fly *Ph. papatasi* possesses salivary anticoagulants which have an apyrase activity, but lack an erthyma-inducing substance (EIS) (Alder & Theodor 1926; Riberio 1987). On the other hand the New World sand fly *Lu. longipalpis* feeds by capillary action and possesses antiplatelet activity, an apyrase, and an erthyma-inducing substance (EIS). The presence of apyrase and EIS factors, together with the short mouth parts, helps *Lu. longipalpis* to feed on the blood released from the superficial blood capillaries. Treatment with trypsin or heating for one minute in boiling water destroys the activity of EIS and suggests that EIS is a peptide (Riberio et al. 1986).

Crop contents and their function

Gas bubbles of aspirated oxygen are present inside the crop of mosquitoes and sand flies. The complete absence of carbon dioxide in them indicates that no fermentation occurs in the crop (Fisk 1950). Although these bubbles are present in the crop and also in the midgut of unfed and sugar fed sand flies (Lewis 1974); no information is available about their chemical nature. The pH of the crop contents of *Culex pipiens* (Say), *Ae*. *aegypti*, and *An*. *quadrimaculatus* (Say) is acidic compared to the basic pH of its midgut (MacGregor 1931).

In *Lu*. *longipalpis* the final destination of the fluid meal is a function of the method of feeding rather than the chemical composition of the fluid. Thus, sugar solution goes directly into the crop and the blood meal, which is imbibed through a membrane, goes to the midgut (Ready 1978). In black flies the sugar solution is not routed to the crop in all flies, in some it goes to the midgut (Disney 1970).

Different enzymes secreted during blood digestion

The pH of the midgut is not easily determined in blood fed females because mammalian red blood corpuscles have a buffering capacity which over masks the gut pH, whereas the midgut epithelium cells remain slightly acidic in nature

(Fisk 1950). No such studies about the pH of midgut and crop contents of sand flies are available.

Different types of serine proteases are secreted before and after a blood meal by the midgut epithelial cells. These proteases are classified by activity and pH optima (Gooding 1975). Serine proteases are present in mosquitoes like Ae. aegypti and Cx. nigripalpus (Graf & Briegal 1985; Borovsky 1986). To understand their mode of action one has to answer the following questions: (1) What is the state of enzyme synthesis prior to the intake of a blood meal? (2) What initiates the rise in enzyme synthesis? (3) What is the relationship between the size of the blood meal and the amount of trypsin- and chymotrypsin-like enzymes secreted? (4) What is the relationship in the amount of total protein in the blood meal and the quantity of the secreted enzymes? (5) What is the effect of blood meal on the changes in the pH of the midgut? (6) What is the effect of proteolytic enzyme inhibitors on the rate of blood digestion? and (7) What effect proteolytic enzymes have on the growth of endoparasites and how the parasites withstand the changes in the midgut pH values?

In mosquitoes, ingestion of a blood meal follows within first 30-35 minutes by rapid diuresis (Boorman 1960; Williams et al. 1983; Mahmood & Nayar 1989). Several changes occur in the structure of the secretory cells of the midgut as described previously. These changes are accompanied by synthesis of serine proteases by midgut epithelium. The enzymes found in the midgut are active; however, synthesis of inactive zymogen that is later secreted into the midgut and activated there cannot be discarded. Although there is no example in which insects secrete an inactive zymogen, the digestive secretory cells of Ae. aegypti contain very few zymogen grannules per cell compared to vertebrate midgut (exocrine cells) and trypsinogen might occur in the digestive cells of Ae. aegypti (Graf & Briegal 1985).

The midgut of mosquitoes has only one type of secretory cells and absorptive cells are absent. The function of absorption is also performed by the secretory cells. They have well developed microvilli, an elaborate basal labyrinth, and many mitochondria in the basal and apical region of the cell. The secretory cells have few secretory granules per cell (Graf et al. 1986). In Ae. aegypti both m-RNA and trypsin are synthesized after the ingestion of a blood meal (Gooding 1973). Alkaline proteases are detected 4 hrs after blood feeding and reach their peak activity at 24-35 hrs depending on the mosquito species (Gooding 1966a, 1966b, 1975; Briegal & Lea 1975; Borovsky 1986; Graf et al. 1986).

The pH optima for inset proteolytic enzymes such as chymotrypsin, aminopeptidases, carboxypeptidase-A and carboxypeptidase-B are alkaline. Different substrates and inhibitors are used to characterize these enzymes (Gooding 1969). Trypsin and chymotrypsin are the major enzymes present in the midgut of mosquitoes *Ae*. *aegypti*, *Culex fatigans* (Weid), goat lice *Melophagus* *ovinus* (L), and human lice *Pediculus* *humanus* (L). Both enzymes are absent in the midgut of blood fed bed bug *Cimex* *lectularius* (L), and kissing bug *Rhodinus* *prolixus* (Stal). These insects contain a high molecular weight protease that is active at pH 5 (Gooding 1969). Gooding (1969) also suggested that insect trypsin and chymotrypsin probably have serine and histidine at their active centers. The amount of trypsin secreted after a blood meal is directly correlated to size of blood meal (Gooding 1973; Graf & Briegal 1985). A similar relationship was shown between the size of blood meal and the amount of total proteases (Shambaugh 1954). The midgut and gut lumen of tsetse fly *Glossina* *morsitans* (Westwood) shows at least 6 different types of proteolytic enzymes (Gooding & Rolseth 1976).

The rate of trypsin-like enzyme secretion in certain hematophagous insects such as horn fly *Haematobia* *irritans* (L) and stable fly *S*. *calcitrans* depends on the ambient temperature (Honda et al. 1985). In blood fed *H*. *irritans* trypsin-like activity is detected earlier at 30°C than at 25°C. The rate of protein digestion is 2 fold faster at 25°C than at 30°C (Honda et al. 1985).

The rate of blood meal digestion in sand flies depends on the source of the blood meal. Schlein et al. (1982a, 1982b, 1983) reported vector incompatibility between field collected _Ph_. _papatasi_ and _Le_. _major_ due to the presence of turkey blood meal. They also showed that if sand flies were fed leishmania parasite in saline 24 hrs prior to a blood meal, the rate of infection was low (Schlein et al. 1983). This observation was contrary to the earlier studies in which sand flies showed a high rate of infection even when they were fed on saline containing promastigotes of a local strain of _Le_. _tropica_ (Adler 1938).

Serine proteases and their mode of action

Structure of serine proteases.

Serine proteases are a large group of enzymes with a serine residue at their active site. These enzymes are irreversibly inhibited by diisopropylflurophosphate (DFP). DFP is a flouridated phosphate ester and is cleaved by trypsin or chymotrypsin. The enzymes are classified as endopeptidases because the cleavage of the terminal peptide is inhibited by the charge on the amino or carboxyl group of terminal residue. Chymotrypsin and trypsin are classified as serine proteases. Mammalian enzymes are secreted in the form of zymogen or proenzyme, an inactive form of an enzyme. Mammalian trypsin and chymotrypsin are synthesized as trypsinogen and chymotrypsinogen at the pancreas endoplasmic reticulum (Stryer 1975). The enzymes are then transferred to the

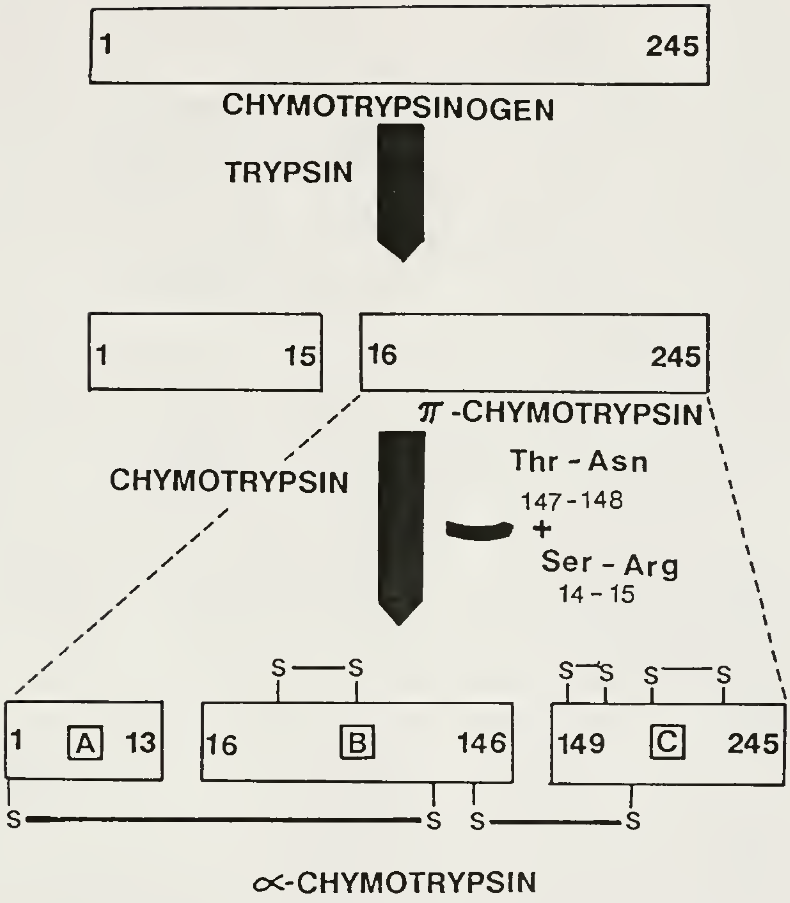

Figure 2-4. Diagramatic representation of the activation of chymotrypsin resulting in the formation of α chymotrypsin (adapted from Stryer 1975).

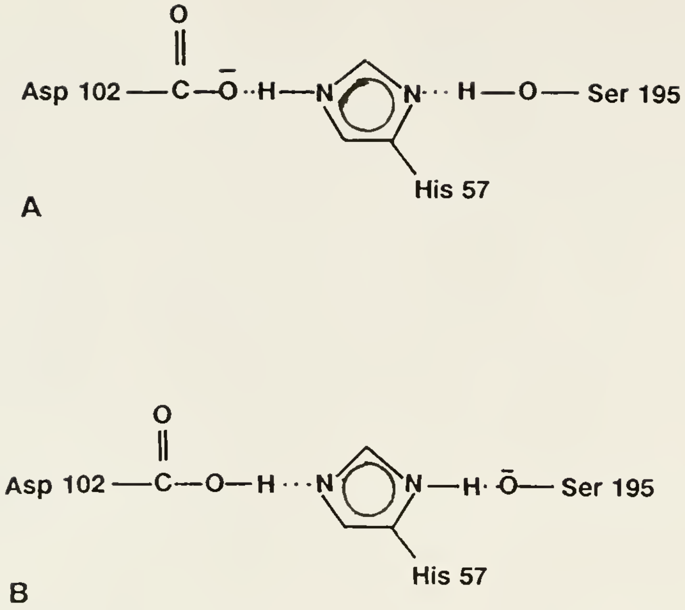

Figure 2-5. Charge relay system of chymotrypsin molecule. A = normal charge distribution in a chymotrypsin molecule; B = Conversion of chymotrypsin into a powerful nucleophile. The removal of one proton by aspartate 102 from serine 105 via histidine 57 results the conversion of serine 195 into a powerful nucleophile (adapted from Stryer 1975).

golgi apparatus where a lipid membrane is secreted around them. Membrane bound trypsinogen and chymotrypsinogen are transported into the gut lumen. The mammalian chymotrypsinogen molecule has 245 amino acid residues. It is activated by the action of trypsin that brings about cleavage of the molecule at arginine 15 and isoleucine 16. The resulting π-chymotrypsin is completely active but is unstable. It is further cleaved by the action of other π-chymtrypsin molecules into α-chymotrypsin, a stable molecule (Fig. 2-4).

The mammalian α-chymotrypsin molecule has three peptide chains which are interconnected by two disulfide bonds (Fig. 2-4). It has a compact ellipsoidal shape and all the charges are on the surface of the molecule except the three most important charges that play a crucial role in its activity. These residues are serine 195 (Ser), histidine 57 (His) and aspartic acid 102 (Asp). Serine 195 is a strong nucleophile and plays an active role in the reactivity of both trypsin and chymotrypsin.

The high nucleophile activity of the serine 195 is contributed to the presence of charge relay system between serine 195, histidine 57 and aspartic 102 (Fig. 2-5).

Mode of action of serine proteases. The serine proteases hydrolyze peptide bonds by attacking the carbonyl group of the peptide bond through the oxygen atom of the hydroxyl group of serine 195. This reaction is brought

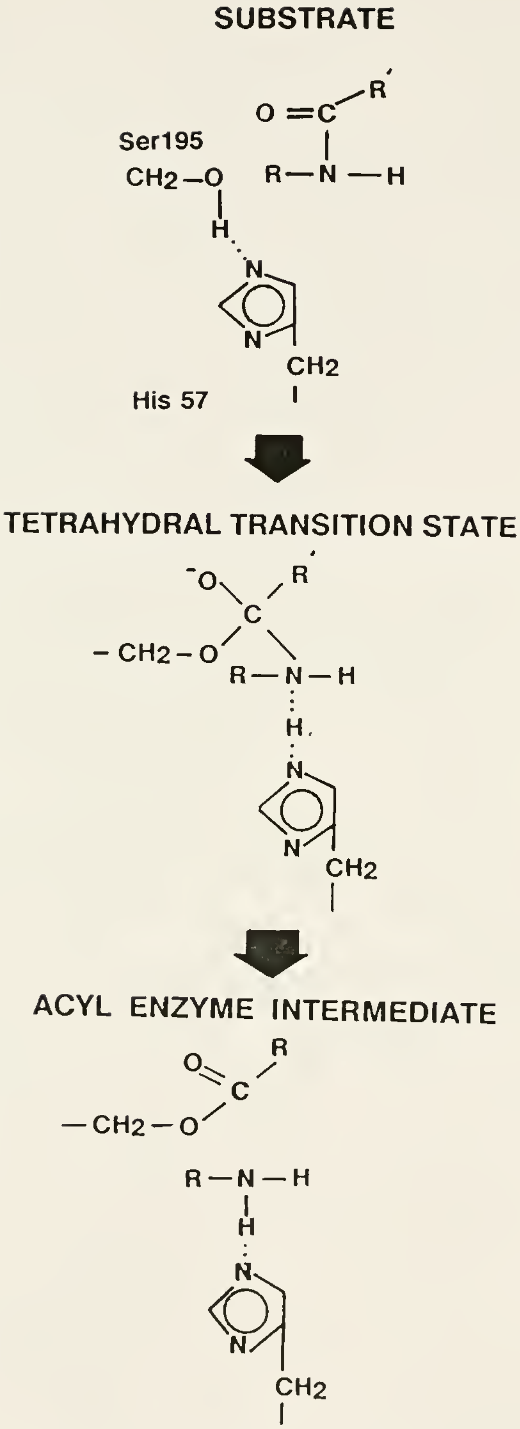

Figure 2-6. Acylatation reaction during the hydrolysis of a peptide bond by chymotrypsin (adapted from Stryer 1975).

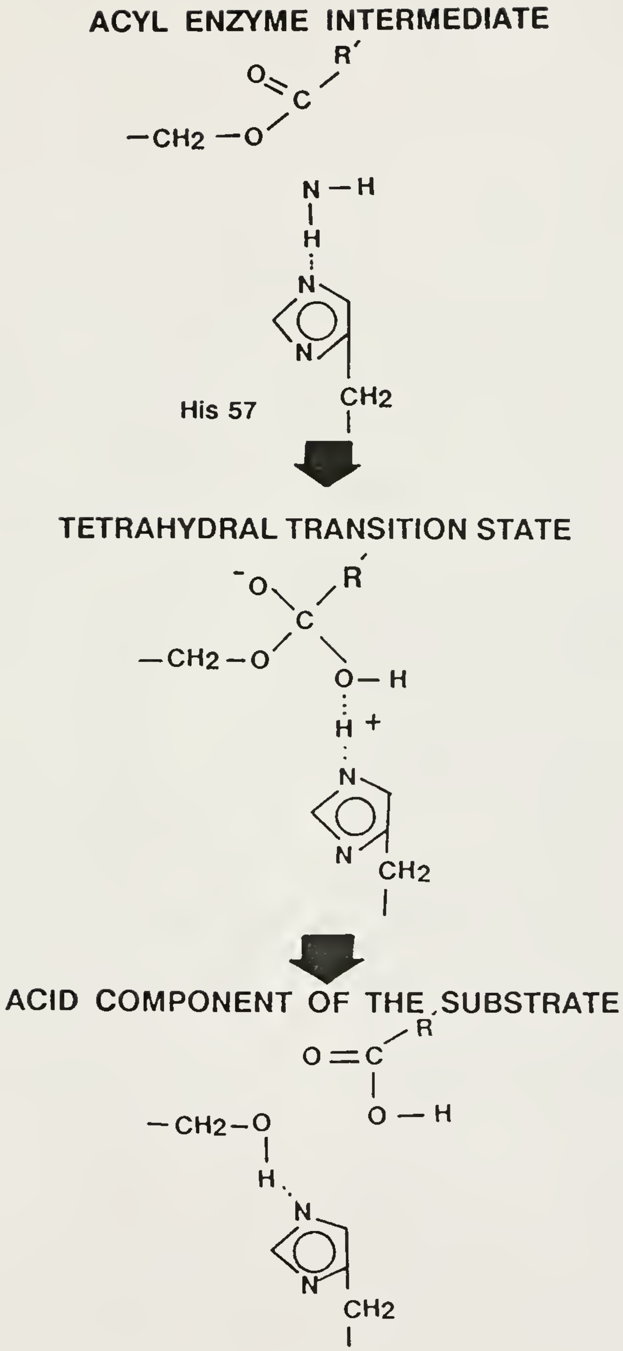

Figure 2-7. Deacylation reaction during the hydrolysis of a peptide bond by chymotrypsin molecule (adapted from Stryer 1975).

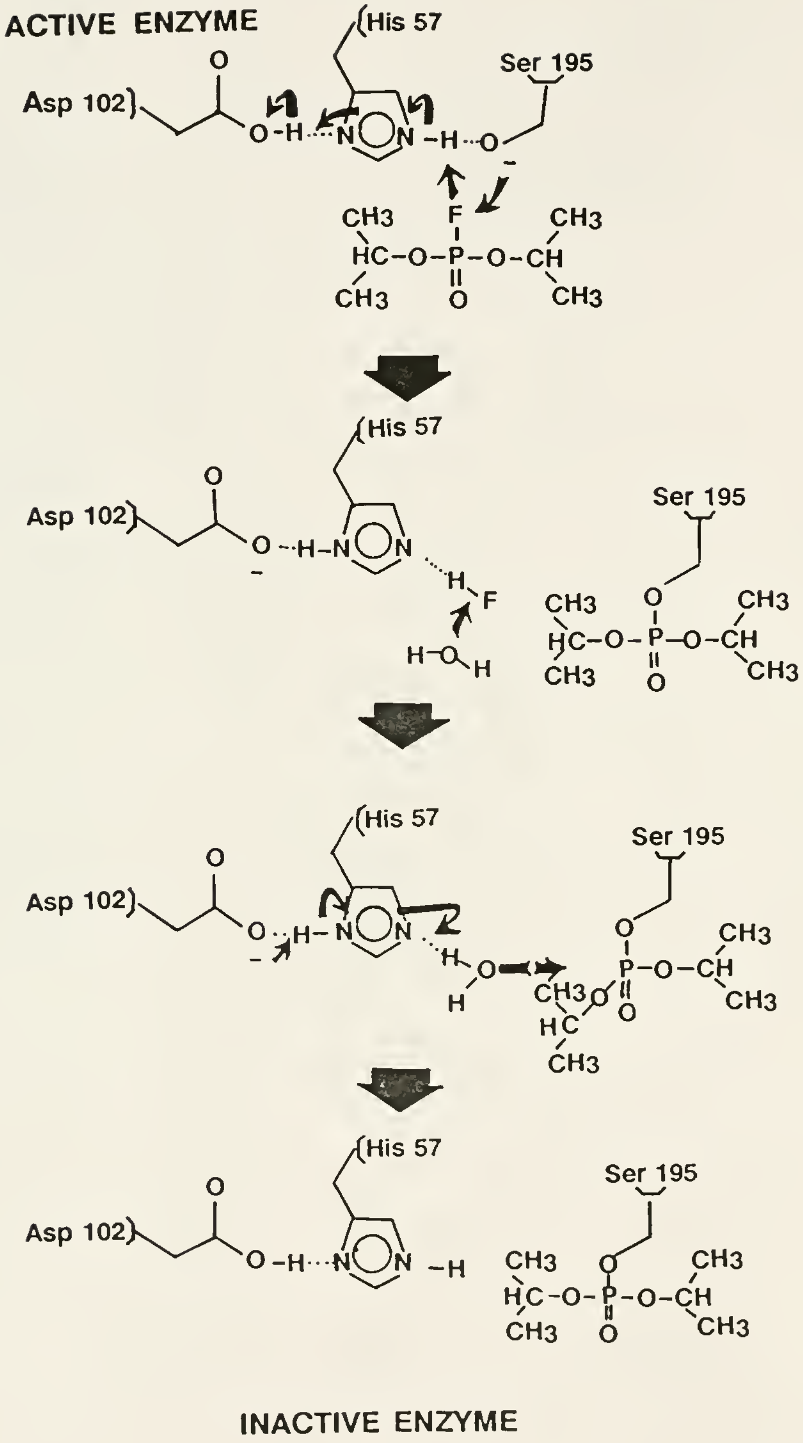

Figure 2-8. Irreversible deactivation of trypsin-like enzyme by DFP.

about by a charge relay system that helps to draw a proton from the hydroxyl group of serine 195 (Fig. 2-5). The nitrogen atom of the susceptible peptide bond then receives a proton from Histidine 57 and the peptide bond is cleaved. This brings about the completion of the acylation stage of the hydrolytic reaction and a complex results in which the amine component is hydrogen bonded to histidine 57, and the acid component of the substrate is esterified to serine 195 (Fig. 2-6).

In the next step a water molecule is added resulting in several steps of proton removal and ionic attack of the carbonyl carbon of the acyl group (Fig. 2-7). As a result the active site is regenerated.

Inhibitors of digestive enzymes. Certain compounds act as proteinase inhibitors, such as certain factors in the serum from mammalian blood can cause *in vivo* inhibition of *Ae. aegypti* proteinase (Fisk & Shambaugh 1952; Gooding 1966a, 1966b, 1972, 1974; Huang 1971). Certain other compounds, DFP (Irreversible inhibitor of both trypsin and chymotrypsin) binds irrevesibly to serine 195 at the active site (Fig. 2-8), TPCK (tosylamide-2-phenyl chloromethyl ketone; chymotrypsin inhibitor) and TLCK (tosyl-L-lysine chloromethyl ketone HCl; trypsin inhibitor), have been used for qualitative and quantitative determination of proteolytic enzymes (Graf & Briegel 1985; Brovosky & Schlein 1987; Brovosky & Schlein 1988).

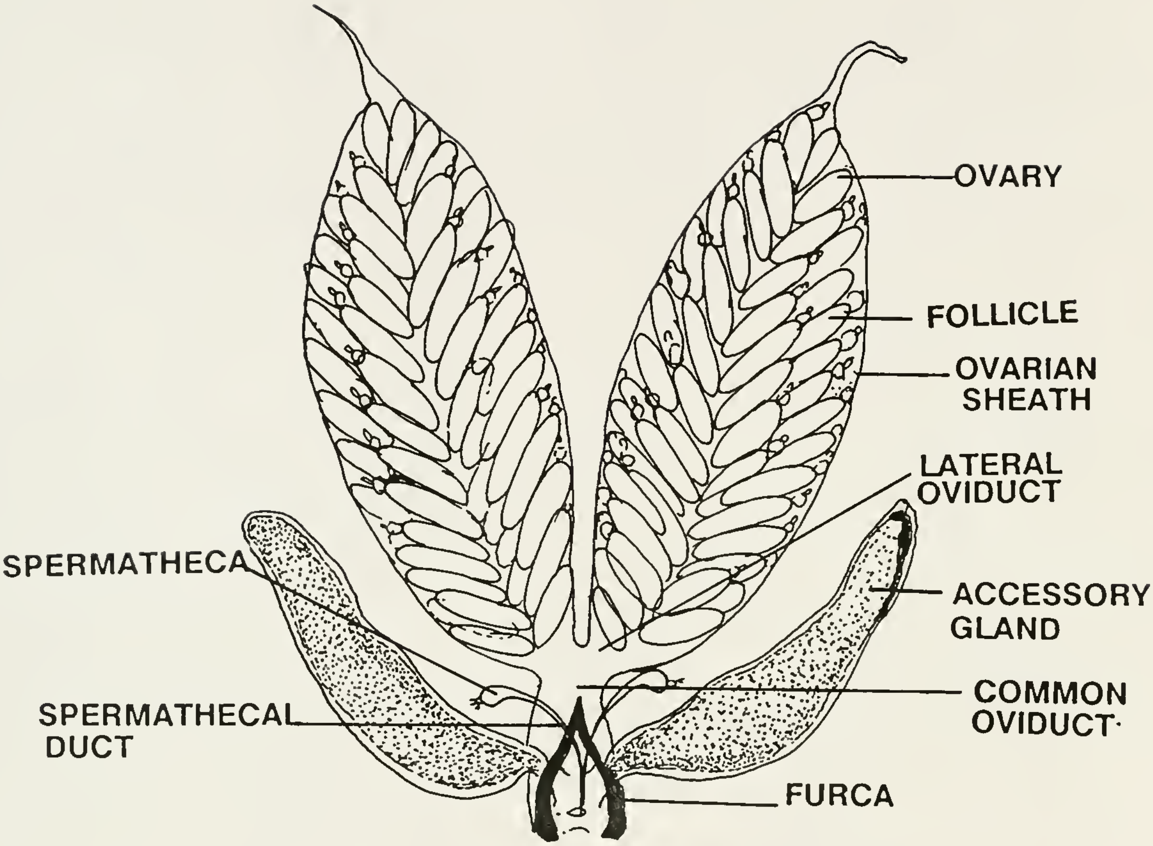

Figure 2-9. Diagramatic representation of the reproductive system of a female sand fly.

Female Age Grading

Reproductive System of Female Sand Flies

The reproductive system of female sand flies consists of a pair of fusiform ovaries, each of which has a separate lateral oviduct that opens into a common oviduct. The latter is a short, wide tube lined with cuticle and which has circular and longitudinal muscles on the outside. The common oviduct opens into a wider portion, or vagina, and is surrounded by arms of a chitinous furca (Perfil'ev 1968). Two elongated accessory glands are present on the sides of the ovaries and also open into the vagina. In newly emerged females, these glands are pale, transparent, and lack granular substance, whereas the granular substance is present in fed and/or parous females (Lewis 1965; Lewis et al. 1970) (Fig. 2-9). These granular secretions are necessary for protecting eggs against fungal and bacterial infections (Adler & Mayrink 1961).

Two spermathecae open on the sides of the bladder-shaped vagina by their respective spermathecal ducts in some species (Sinton 1925; Sinton & Braud 1928) or by a common spermathecal duct in others (Sinton 1925). In the majority of the Diptera, three spermathecae and three spermathecal openings are the primitive condition. This condition corresponds to the aedeagal openings in the male. The female spermathecal openings are coadapted to the aedeagal openings of the male (Downes 1968). The size of the spermathecal duct varies in different species of sand flies. The spermathecae are of variable shapes and are useful in the identification of different species of sand flies (Young 1979).

Downes (1968) considered the presence of two spermathecal ducts, which open separately a primitive character compared to a common spermathecal duct and a single spermathecal opening. Gerber (1970) studied the evolution of spermatophores in Pterygotan insects. He considered the presence of spermatophore as the most primitive condition and the deposition of accessory gland secretions, together with sperms directly inside the spermathecae and/or bursa copulatrix, or vagina, as the most advanced character. The presence of a mating plug formed by the accessory gland substance and the sperms (a condition found in some mosquitoes) was considered as an intermediate character between the above two conditions.

Each spermatheca has a round or conical head, present at the apex of the spermathecal neck. The head is surrounded by numerous gland cells which open into the neck of spermatheca by different number of chitinous canals. The gland cells form a large rounded gland that can be easily seen in fresh preparations. It is destroyed by fixation processes using alcohol or KOH (Perfil'ev 1968). The gland roduces a fluid into the spermathecal head that serves to

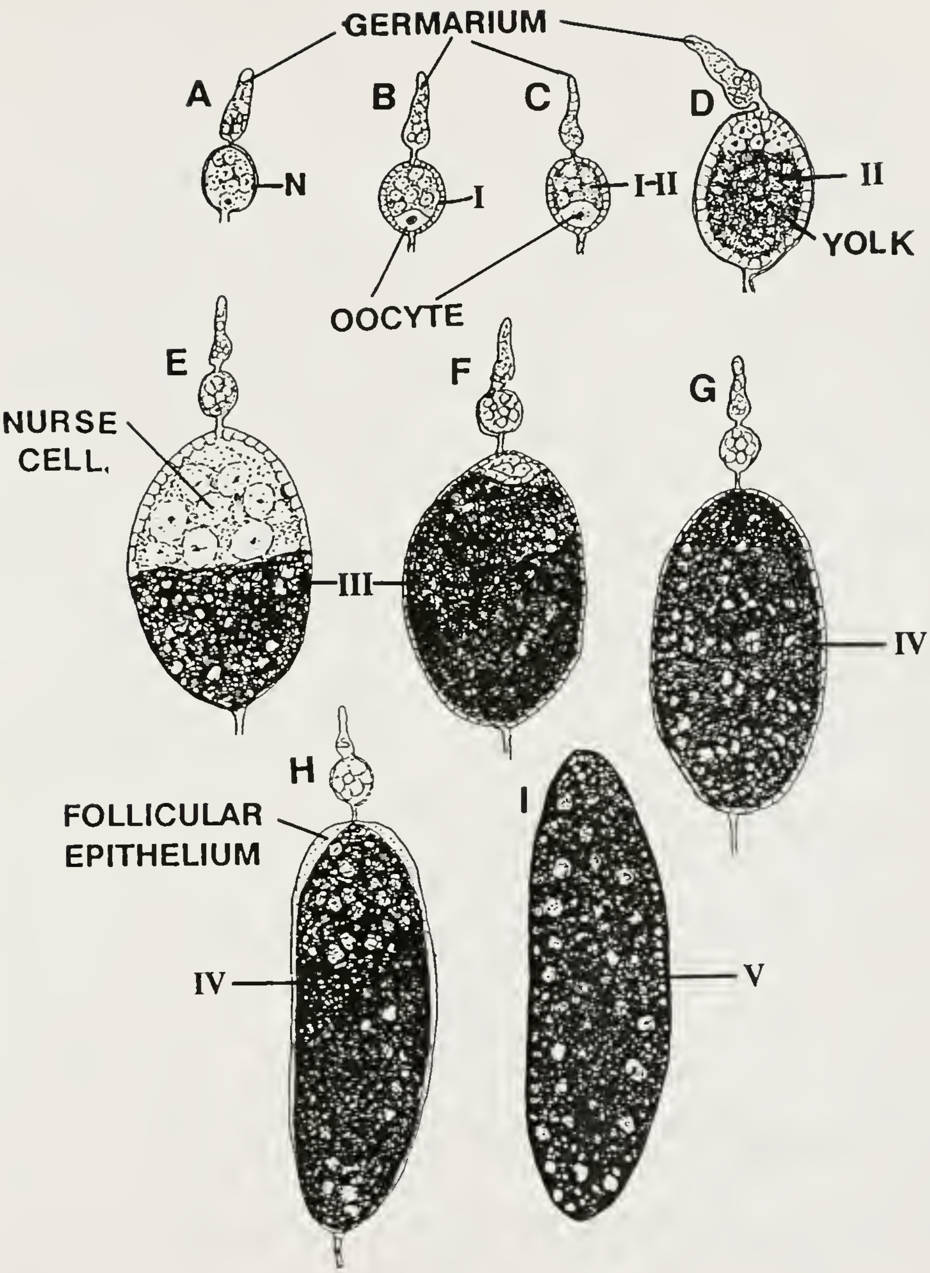

Figure 2-10. Different stages of the follicular development in Lutzomyia anthophora. A = stage N; B = stage I; C = stage I-II; D = stage II; E-F = stage III; G-H = stage IV; and I = stage V.

preserve the spermatozoa (Perfil've 1968). The vagina narrows down to a tube that opens to the outside as the vulva. The vulva is situated near the anus and is separated from it by a muscular thickening.

The ovaries are enclosed in an ovarian sheath that continues with the oviduct on lower end and on the upper end forms a soft filament like structure (Fig. 2-9). The filament or ligament of each ovary connects it to the body cavity (Fig 2-2). The ovaries are of polytrophic meriostic type. Each ovary has a variable number of ovarioles, radially attached to the internal oviducts on each side. The part of the internal oviduct where the pedicle of the ovariole attaches to the oviduct is called the calyx. Each ovariole is attached to the ovarian sheath by a filament and has its own ovariole sheath. The part of the ovariole where active cell division occurs is called the germarium which has a variable number of oogonia. Each developing follicle (e.g., in Ph. papatasi) contains about seven nurse cells and an oocyte (Jobling 1987). Each follicle, present in its own follicular tube, has its own follicular epithelium. At a given time, at least three follicles are present in different stages of development. The size of the follicles depends on their stage of development (Fig. 2-10).

In gonoactive females, the last follicle in each follicular tube begins to develop after the ingestion of a blood meal. The follicles of newly emerged females contain eight monomorphic nuclei, seven of which are nurse cells and the remainder is the oocyte or ovum. The function of the nurse cells is to nourish the ovum. The development of the follicle was first described in anopheline mosquitoes by Christophers (1911), who divided the whole developmental cycle into stages I-V. Mer (1936) recognized two more stages in the initial growth period of the follicle and called them as N and I-II (Fig. 2-10)

Detinova (1962) described the following stages of ovum maturation and which are essentially as applied to sand flies due to similarities in their growth pattern.

Stage N. A spherical follicle that consists of 8 monomorphic cells (Fig. 2-10-A).

Stage I. The follicle is spherical or slightly oval. Seven nurse cells lie above a distally located oocyte (Fig. 2-10-B).

Stage I-II. The follicle is distinctly oval in shape and one or two rows of yolk granules encircle the oocyte nucleus (Fig. 2-10-C).

Stage II. The egg grows larger due to accumulation of large amount of yolk granules around the oocyte nucleus (Fig. 2-10-D). The ovum is considerably larger than the nurse cells and occupies half of the follicle.

Stage III. The follicle is oblong in shape and the nucleus is hidden by the yolk granules that cover 1/2 to 3/4 of the follicle (Fig. 2-10-E & 2-10-F).

Stage IV. The follicle is longer and larger and the nurse cells occupy only the upper most part of it. More than 9/10 of the follicle is occupied by oogonium, that is full of yolk (Fig. 2-10-G & 2-10-H).

Stage V. The nurse cells are pushed towards the tip of the follicle due to increased yolk deposition. The follicle is covered with a well developed chorion. A micropyle is formed at this stage (Fig 2-10-I). In certain species of mosquitoes the nurse cells are pushed out of the micropyle.

The above classification of different developmental stages of the follicle has been successfully applied to different species of sand flies (Magnarelli et al. 1984). Certain species of sand flies can develop their eggs autogenously. This characteristic introduces an error in determining the epidemiological importance of such species in the transmission of nontransovarially transmitted pathogens such as _Leishmania_. Autogenous females can maintain transovarially transmitted viruses without blood feeding; e.g., several sand-fly born viruses of vertebrates are transovarially transmitted in their insect hosts (Tesh & Chaniotis 1975; Endris et al. 1983; Tesh & Modi 1983).

Comparision of egg development between autogenous _Ph. papatasi_ held on fructose solution only, with those provided with a blood meal, showed no difference in the total number of eggs produced (Magnarelli 1984). Twenty-two percent of the females were autogenous and produced eggs without a blood meal. The rate of egg development was similar in both autogenous and anautogenous females. Egg development was asynchronous in blood fed females (Magnarelli 1984).

Most species of sand flies are gonotrophically concordant and require only one blood meal for complete development of a clutch of eggs. Certain sand flies, like Ph. papatasi, take blood at any stage of follicular development (Schmidt & Schmidt 1965; Killick-Kendrick 1979; Magnarelli et al. 1984). Multiple feeding during one gonotrophic cycle increases the chances of parasite transmission by infected sand flies (Killick-Kendrick 1979). The effect of a second blood meal on the rate of development of parasites ingested with the first blood meal is unknown. Adler and Theodor (1926) suggested that multiple feeding was a phenomenon which occurred only in the earlier stages of egg development, but Christensen and Herrer (1980) observed a significant number of gravid Lutzomyia species, attracted to animal baited traps. This observation led Magnarelli et al. (1984) to conclude that sand flies would even take a blood meal just prior to oviposition if a host was present.

In anautogenous species of sand flies, such as Lu. longipalpis, fecundity is correlated with the size and composition of the blood meal. Composition of the blood meal differs depending upon the mammalian host species. On the other hand, in autogenous species fecundity is directly related to the larval nutrition (Magnarelli et al. 1984).

Different Methods of Age Grading Sand Flies

Although the following age grading techniques have been used successufuly for mosquitoes, some of these such as presence of parasitic water mites (Corbet 1963; Mullen 1975), appearance of scaling of the wings (Perry 1912), tergal pigmentation patterns (Linley & Baverman 1984), presence of meconium in the midgut (Detinova 1962), presence of green colored fat in the haemocoel, eggs retained in the ovaries, presence of greatly dilated, translucent internal oviduct, size of ampullae, qualitative changes in the shape of the ampullae, and tracheation of ovaries and midgut are not effective for sand flies.

Presence of daily growth layers. Many exopterygote insect species such as grasshoppers, flies, and mosquitoes deposit cuticular growth layers on their exoskeleton. Similarly, these are deposited as bands on internal muscle apodemes of thoracic phragmata in endopterygotes. These layers have been used to age grade populations in the field (Neville 1963a, 1963b, 1963c, 1983; Schlein & Gratz 1972; Tyndale-Biscoe & Kitching 1974; Ellison & Hampton 1982; Yual & Schlein 1986).

The growth layers were initially described as fluorescent layers in the resilin of locust-rubber like cuticle (Neville 1963a, 1963b). They appear as dark and light bands under phase contrast microscopy or crossed polaroid light filters (Neville 1963c). The endocuticle deposited during the day has its microfibrils oriented in one direction, whereas that deposited during the night is helicoidal. Thus, night deposited cuticle appears dark and day deposited layer is light in color. A consecutive pair of cuticular layers (appearing as a dark a light band by crossed polaroids light) represents 24 hrs growth in grasshoppers (Neville 1963c). The growth layers are easy to observe in insects with melanized cuticles, but special staining methods are required in insects with transparent apodemes (Schlein & Gratz 1973; Schlein 1975; Schlein 1979a, 1979b; Yual & Schlein 1986; Alexander 1988).

In Glossina one day error was possible in counting of the growth layers. The width of growth layers decreases in older flies but is easy to count at 40X magnification (Schlein 1979a). The banding is dependent on the fluctuations in the daily temperature and cuticular growth is known to cease completely at low temperature (Neville 1965; Schlein 1979b). Banding is most clear when the daily temperature is below the threshold of cuticular growth of an insect (Tyndale-Biscoe & Kitching 1974). In most insects cuticular growth layers are formed immediately after emergence and if the anomalies are taken into account, this method is satisfactory for determining age of both sexes.

This method was successfully applied for age grading wild caught sand flies (Yual & Schlein 1987; Alexander 1988).

Accessory glands. Granular accessory gland secretion was first noted by Adler and Theodor (1935) in *Phlebotomus perniciosus* (Newstead). The accessory gland secretion was secreted after blood feeding and some of it was retained in accessory glands after oviposition. The eggs are coated with it during oviposition and adhere them to the oviposition substrate (Ward 1985).

There are differences in opinion regarding use of accessory gland as indicator of age in wild caught sand flies. Adler and Theodor (1957) considered the presence of accessory gland secretion as indicator of old age. This view was questioned by Garnham and Lewis (1959) who examined field collected sand flies and found that a large proportion of them contained accessory gland substance. They thought that it was possible for nulliparous flies to develop the accessory gland substance. The accessory glands of *Ph. papatasi* also secrete accessory gland secretion after a blood meal but not all females retain part of this substance after oviposition (Dolmatova 1942). Some Panamanian sand flies secrete the accessory gland fluid even before taking a blood meal and are capable of discharging it at various times (Johnson & Hertig 1961). Lewis (1965) noted structural changes of the accessory glands in five species of Lutzomyia from Bleize and concluded that this method was unreliable. The accessory glands of Lu. flaviscutella (Mang) and Lu. furcata (Mang) were also considered unreliable indicators of parity (Ready et al. 1984).

The presence of accessory gland substance together with small ovaries was considered a reliable indicator of old age in some Kenyan sand flies (Lewis & Minter 1960). Soshina (1951, reprint not seen referred from Detinova 1962) used the accessory glands of Phlebotomus and age graded the population by dividing it into four categories, 1) nulliparous and newly emerged females, 2) females in their first gonotrophic cycle, 3) one parous females and 4) females which refed after their first oviposition.

The accessory glands were also used to study the population trends, such as breeding seasons, appearance of successive generations, and the mode of dry-season survival of Sergentomyia garnhami (Minter 1964). Lewis et al. (1970) found that in Lu. shanonni (Dyar), a higher proportion of nulliparous females showed accessory glands secretions which might be due to development of secretions in the glands prior to development of the ovaries. They suggested that some use of the accessory glands of Lu. ovallesi (Ortiz) and Lu. panamensis (Shannon) could be made for the study of general trends of their populations. Chaniotis and Anderson (1967) considered the accessory glands of 3 California sand fly species as very reliable indicators of their parous

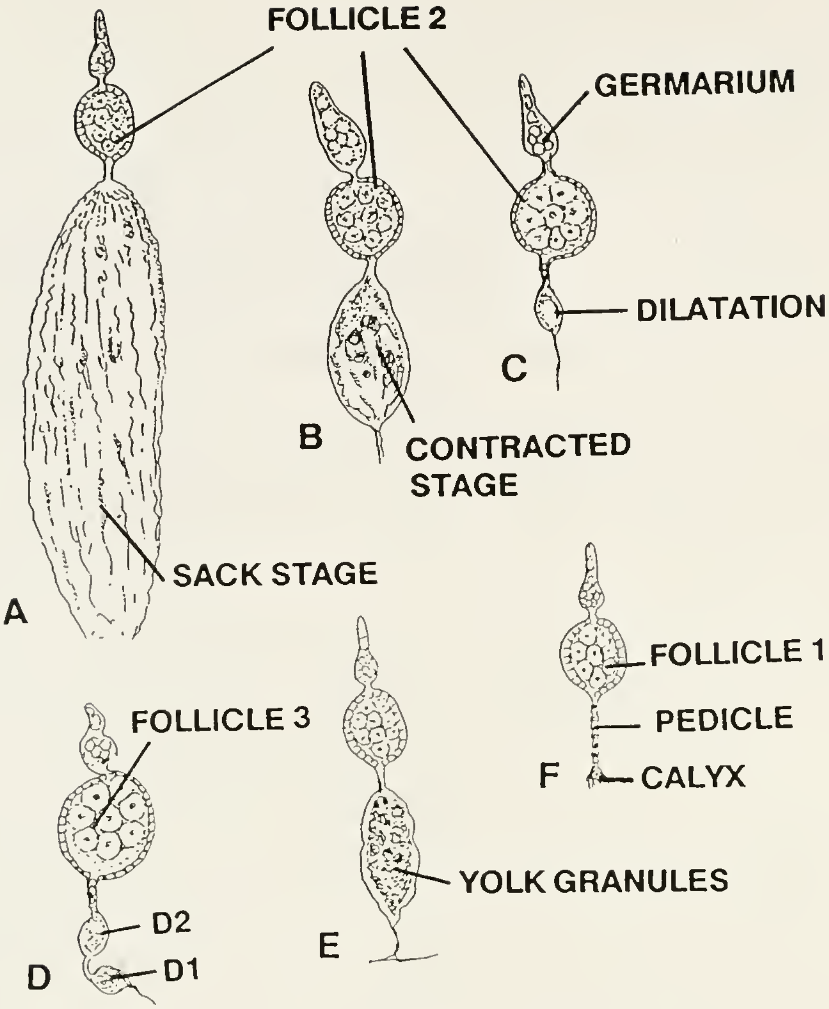

Figure 2-11. Digramatic representation of the different stages that are found during the formation of dilatations. A = an ovarian follicle with a sack stage that is present just after oviposition; B = half contracted sack stage; C = an ovariole of a 1-parous female showing one dilatation; D = an ovariole of a 2-parous female showing 2 dilatations; E = a degenerated ovariole from a gravid female, note the presence of yolk granules; An ovariole from a nulliparous female, note the presence of ovarian tissue or calyx attached to the pedicle.

status. Only 3% of the 300 parous females lacked granular material in their accessory glands.

Ovarian follicular dilatation. This method is applicable to females of any age and stage of blood digestion (Detinova 1962). Ovaries are dissected out in a drop of physiological saline. An ovary is moved to the side of the drop and the ovarian sheath is carefully removed at places. In these areas the ovarioles are very loose and the number of dilatations can be easily counted by stretching the ovarian follicle. In females, recently oviposited, the ovariole are in a sac-like stage (Fig. 2-11-A). The sac-like stage contract with time and form a bead-like structure (Polovoda 1949 reprint not seen referred from Detinova 1962) (Figs. 2-11-B, 2-11-C & 2-11-D).

In each gonotrophic cycle of sand flies a certain number of eggs degenerate. Most of the time the degenerated eggs belong to the same ovariole. In multiparous females which have recently oviposited number of dilatations can be determined from the degenerated eggs (Fig. 2-11-E). The number of dilatations found in the ovaries of a female give an indication of her age. To estimate the female age in different seasons, lengths of the gonotrophic cycles determined at different temperature is required. This method can be used to determine the vertical survivorship of both healthy and infected field collected females in different seasons of the year (Reisen et al. 1986).

Opinions vary concerning the applicability of dilatation technique for age grading sand flies. The ovaries of parous sand flies are very loose (Dolmatova 1942). Detinova (1962) considered the small size of sand flies as the major obstacle for the dilatation technique. Lewis and Minter (1960) applied the dilatation technique to determine the parity of unfed flies from Kenya. They concluded that it was possible to determine the parity of females by this technique. They found a good correlation in the parous females and presence of the accessory gland secretion. On the other hand Lewis (1965) found a certain degree of error in determining parity of wild caught _Lutzomyia_ species by this technique.

Similarly, Ready et al. (1984) applied Polvodova's method to assess the age of the laboratory reared _Lu_. _flaviscutellata_ (Mangabeira) and _Lu_. _furcata_ (Mangabeira). Results of their blind test showed that this method was good for simply classifying females into nulliparous and parous groups.

The dilatation technique was successfully applied to age grade _Lu_. _ylephiletor_ (Fairchild & Hertig). The average age of was greater for females collected in biting collections than those collected from resting sites. This fact was supported by large number of flagellate infections found in females from biting collections (Johnson et al. 1963). Field collected _Ph_. _papatasi_ from Iran showed only one-parous and nulliparous females. No multiparous female were caught (Magnarelli et al. 1984). Chaniotis and Anderson (1967) considered age grading by dilatations as an impracticle and time consuming technique. On the other hand, Wilkes and Rioux (1980) found dilatation technique very useful. They utilized this technique to compare the age structure of Ph. ariasi (Tonnoir) collected from the walls of a farm house and in light traps. A significant difference was found between ages of flies collected by the two methods. They concluded that for epidemiological studies, light trap collections were better. About 1/3 of the females in light trap collection were one-parous and few were two and three parous. The collections from the wall of the farm house included only one female with one dilatation and rest of 105 females were nulliparous.

Application of the dilatation technique requires some caution because of false dilatations formation in nulliparous Culex mosquitoes (Yajima 1970; Oda et al. 1978; Nayar & Knight 1981; Reisen et al. 1986). In Culex tritaeniorhynchus (Giles) the tracheolar coiling method gave a better indication of nulliparity and was recommended for use on one ovary and the dilatation technique on the other ovary (Reisen et al. 1986). In laboratory control experiments, the greatest difficulty was encountered in distinguishing 2 parous gravid females; it was always much easier to find the degenerated eggs in the field collected mosquitoes as compared to the laboratory collected controls (Reisen et al. 1986). During laboratory control studies, experimental conditions prevented the females from refeeding on the night of oviposition and might had contributed to the discrepancy (Reisen et al. 1986). Gillies and Wilkes (1965) and Spencer (1979) also cautioned against errors associated with formation of dilatation in laboratory experiments. No studies are available describing formation of false dilatation in sand flies.

Hormonal Control of Insect Reproduction and Role of Juvenile Hormone III in Adult Insects

Juvenile hormones (JH) are synthesized and released in insects from the corpora allata. In larval instars of insects JH and ecdysone act concurrently. Ecdysone triggers the biosynthetic pathways essential for molting. During morphogenesis JH act in concert with ecdysone. Juvenile status in insects depends upon continuous presence of JH, that act on the cells and prevent them from maturing. In immature insects JH manifests a multiplicity of morphogenetic effects. It control the kind of cuticle the epidermal cells secrete in response to ecdysone. Juvenile hormones also influence the development of internal organs like central nervous system, gonads, and midgut, where they deter maturation and metamorphosis (Sehnal 1968). Juvenile hormones also hinder the maturation of the imaginal discs which are the primordia for many adult integumentary structures in endopterygote insects.

In nature, adult mosquitoes are ready to feed on nectar after their cuticle hardens. An active search for sugar feeding begins later due to locomotor activity and receptor sensitivity (Meola & Readio 1988). Mosquitoes take blood to utilize the proteins for egg maturation. Host seeking does not depend on JH in *Ae. aegypti* and is apparently related to maturation of antennal chemosensory afferent neurons involved in detection of lactic acid (Bowen & Davis 1989). In contrast, in *Cx. pipiens*, JH has been reported to control the initiation of biting behavior (Meola & Petralia 1980; Meola & Readio 1987).

Newly emerged female mosquitoes become reproductively competent due to a chain of events that starts by the release of JH from their corpora allata. Juvenile hormone stimulates the primary follicles of newly emerged females to grow in size to a previtellogenic resting stage (Lea 1963; Gwadz & Spielman 1973). The level of JH remains constant in newly emerged and sugar fed *Cx. pipens*; but in *Ae. aegypti*, the JH level is high during the first two days after emergence and then gradually declines. This may be due to feed back inhibition by resting stage ovaries (Rossignol et al. 1981; Shapiro et al. 1986). The latter scientists (1986) observed that in *Ae. aegypti* JH levels dropped three days after emergence with no corresponding increase in esterase levels. They suggested that formation of resting stage follicles in 3-day-old mosquitoes might inhibit hormone production by the corpora allata.

Implantation of both preresting and resting stage donor ovaries into newly emerged females inhibited growth of the follicles of the host ovaries. However, implantation of resting stage ovaries only inhibited growth of the preresting stage host ovaries (Meola & Readio 1988). Absence of JH due to allatectomy shortly after emergence of _Ae_. _aegypti_ and _Cx_. _pipiens_ inhibited the growth of primary follicles to the resting stage (Gwadz & Spielman 1973; Lea 1963).

Application of synthetic JH stimulated the growth of primary follicles (Spielman 1974). Once the resting or previtellogenic stage of the follicle was achieved and the follicle was 50-75 µm in length; no further growth of follicles occurred (Meola & Readio 1988). The follicles of _S_. _calcitrans_ did not develop when females were allatectomized within 1 hr of emergence, regardless if they were provided a sugar or a blood meal. Normal follicular growth is achieved only after implanting of corpora allata (Moobola & Cupp 1978).

A humoral factor released from the brain was thought to be essential for mosquito egg development (Clements 1956; Gillet 1956). Release of this factor was suggested to occur after blood feeding due to the distention of midgut wall.

Midgut distension stimulated the neurosecretory cells of the brain which secreted the brain hormone. The brain hormone directly or indirectly activated the corpora allata. The corpora allata secreted JH which initiated vitellogenesis (Larson & Bodenstein 1959). The above hypothesis was disputed by Bellamy and Bracken (1971), who showed that in Cx. pipiens, only certain proteins initiated egg development instead of sustained stretching of midgut receptors. A brain hormone "egg development neurosecretory hormone" (EDNH) from the median neurosecretory cells (MNC) was also released after eclosion (Lea 1963, 1969, 1972; Meola & Lea 1972) and was stored in the corpus cardiacum (CC). EDNH was released into the hemolymph after blood feeding. Its release is triggered by corpus cardiacum stimulating factor (CCSF) (Borovsky 1982).

Yolk is deposited in sugar fed mosquitoes of several species either by injecting or feeding them high concentrations of 20-OH-ecdysone (Spielman et al. 1971). Spielman et al. (1971) considered the effect a pharmacological artifact. During the same period it was shown that in Ae. aegypti vitellogenin was synthesized by the fat body cells (Hagedorn & Judson 1972). Presence of the ovaries was necessary for vitellogenin production. Fat bodies of sugar fed females synthesized vitellogenin during in vitro incubation with ovaries of blood fed females (Fallon et al. 1974). This led Hagedorn (1974) to suggest that vitellogenin stimulating hormone (VHS) was secreted by the ovaries. Later, Schlaeger et al. (1974) showed that ecdysteroid levels were higher in blood fed females than in sugar fed females. *In vitro* incubation of fat bodies in the presence of 10^5 M 20-OH-ecdysone also resulted in the production of vitellogenin in levels comparable to those found in blood fed females (Hagedorn 1974). However, Borovsky and Van Handle (1979) were not able to stimulate fat bodies in vitro with 20-OH-ecdysone. It was confirmed that fat bodies removed from sugar fed females produced smaller quantities of vitellogenin (5%) compared to fat bodies removed from blood fed females (Borovsky 1984).

Continuous presence of 20-OH-ecdysone secreted by the ovary was not necessary for the synthesis of vitellogenin. In ovariectomized mosquitoes, accumulation of vitellogenin caused feed back inhibition of vitellogenin synthesis (Borovsky 1981a). To explain the above discrepancies, Hagedorn (1983) suggested that fat bodies of sugar fed females were nutritionally inferior than those of blood fed females and there was a high turnover of 20-OH-ecdysone. Later studies, showed that injection of physiological amounts of 20-OH-ecdysone caused the separation of secondary follicles; continuous infusion of physiological amounts of 20-OH-ecdysone did not bring about vitellogenin synthesis in sugar fed females (Beckemeyer & Lea 1980; Lea 1982).

Injection of physiological amounts of 20-OH-ecdysone into isolated abdomens of autogenous Ae. atropalpus (Coquillet) initiated oogenesis. Egg development occurred when the abdomens were treated first with small amounts of JH (Fuchs et al. 1981). Similar results were obtained with anautogenous females of Ae. aegypti (Borovsky 1981b). Thus, the presence of both JH and ecdysone is necessary for egg development in blood fed mosquitoes. Borovsky et al. (1985) showed for the first time that JH III was produced in blood fed females, and vitellogenic ovaries induced egg development in a nonvitellogenic host only if the nonvitellogenic host was first treated with methoprene. Vitellogenesis was induced in isolated abdomens by 20-OH-ecdysone only if the abdomens were first treated with methoperne.

Two stages of oocyte growth follow after ingestion of a blood meal. The first stage, called the initiation stage, when the oocyte grows from the resting stage to stage II or early stage III (0-50 µm) (Gillet 1956). This stage is independent of brain factors (Gillet 1956, 1957). The second stage, or promotion stage, represents when the oocyte grows from stage III to stage V (>50 - 450 µm) and requires the release of EDNH 4-8 hrs after blood feeding (Lea 1972; Gillet 1957). Greenplate et al. (1985) showed that EDNH is released twice in Ae. aegypti, once before, immediately after blood meal and once 8-hrs after the blood meal. They also suggested that the critical time of release of factors stimulating ecdysteroid production occurs at 4-8 hrs. Greenplate et al. (1985) suggested that the release of ecdysteroid is controlled by EDNH and indicated that the first release of EDNH immediately after blood feeding brings ecdysteroid level to 50-60% of its maximum. Eight hrs later, a second release of EDNH brings ecdysteroid to its maximum level.

Release of JH prepares the primary follicles of mosquitoes to become receptive to brain hormone (EDNH) (Shapiro & Hagedorn 1982) and prepares the fat bodies for 20-OH-ecdysone (Hagedorn 1977). Ecdysone is synthesized by the ovaries and is converted into 20-OH-ecdysone by the fat bodies; it stimulates the fat bodies to produce vitellogenin (Hagedorn et al. 1975). The level of JH declines rapidly just after blood feeding in both Ae. aegypti and Cx. pipens (Shapiro et al. 1986; Readio et al. 1988).

Different changes occur in the morphology of the follicular epithelium soon after ingestion of a blood meal. In mosquitoes such as Ae. aegypti, pits or wells form in the surface of the oocyte. The pits later pinch off from the follicular membrane and carry yolk proteins into the oocyte and fuse to form large yolk bodies. Thus, the transport of yolk proteins occurs by pinocytosis (Roth & Porter 1964).

Follicle cells of Rhodnius prolixus lose fluid and shrink as a result of the action of juvenile hormone.

Large spaces appear between adjacent follicle cells resulting in a direct contact between the vitellogenin in the haemolymph and the oocyte surface (Pratt & Davey 1972).

Similarly, in cockroaches, the presence of JH induces appearance of intercellular spaces in the follicular epithelium of the ovary (Bell & Barth 1971). In Leucophea maderae (L), JH also stimulates DNA synthesis in the follicular epithelium of the terminal follicle (Koeppe & Wellman 1980).

In Ae. aegypti, the secondary follicle separates from the primary follicle within 20-hrs after blood meal and is presumably regulated by a rise in the titer of 20-OH-ecdysone (Beckemeyer & Lea 1980).

In Cx. pipens, the synthesis of JH declines rapidly after blood feeding; whereas in sugar fed females of the same age it remains high. Rising ecdysteroid levels may act on the corpora allata and suppress their activity (Rosignol et al. 1981). However, Meola and Readio (1988) suggested that a drop in the level of JH few hours after blood feeding was faster than the increase of ecdysteroid. Shapiro et al (1986) failed to find a direct correlation between JH esterase activity and JH concentration, and they were unable to explain the rapid decline in the level of JH after blood feeding, without an increase in JH concentration.

In Ae. aegypti, an increase in JH III biosynthesis occurred from eclosion to 144-hrs after emergence (Borovsky et al. 1989). When synthesis of JH III was followed *in vivo* at different time intervals after blood feeding, an increase in the synthesis of JH III was found within 2-hrs after blood feeding, followed by a decline in JH III synthesis. Juvenile hormone III concentrations were similar to those found in newly eclosed females. The decline in JH III synthesis was followed by a gradual increase which reached a maximum 46 to 48-hrs after the blood meal. Minimum amount of JH III was synthesized 120-hrs after the blood meal (Borovsky et al. 1989).

Metabolites of JH III were followed in sugar fed mosquitoes and in females at different times after blood meal ingestion (Borovsky et al. 1989). These authors showed that JH was metabolized first to JH-acid and then immediately to JH-diol-acid by epoxide hydrase. JH-acid was a better substrate then JH III for the enzyme epoxide hydrase which hydrated it 24 times faster then JH III (Borovsky et al. 1989).

In *Cx*. *pipiens*, the secondary follicles grow up to 43 µm in length within the first three days after a blood meal. Further growth of the follicle, to 57 µm in length, requires the presence of JH secreted from 4th to 8th day after the ingestion of a blood meal (Meola & Readio 1988).

In *An*. *quadrimaculatus*, the secondary follicles grow up to 85 µm long after ingestion of a blood meal. After oviposition, the secondary follicles develop to the resting stage that average 105 µm long (Meola & Readio 1988). On the other hand, previtellogenic growth of the secondary follicles of An. stephensi is completed during the first gonotrophic cycle, before the ovipostion of the primary follicles (Redfern 1982).

Fat bodies of mosquitoes lose their ability to respond to 20-OH-ecdysone 20-hours after the blood meal (Bohm et al. 1978). The fat bodies may require another burst of juvenile hormone to produce a second batch of eggs (Hagedorn 1983). These observations are also supported by the studies of Readio and Meola (1985). Who allatectomized Cx. pipiens females after their first oviposition, refed them, then noted the length of their secondary follicles. Only a small percentage of the mosquitoes refed and of those that fed, very few had vitellogenic ovaries. They also measured the length of secondary follicles of mosquitoes with intact corpora allata. Their results indicated that JH became available for the growth of secondary follicles 24 hrs after a blood meal. The resting stage follicles were 70 µm in length. The resting stage secondary follicles were competent to complete a second gonotrophic cycle as soon as the mosquito took another blood meal after oviposition. Egg retention beyond the normal gontrophic cycle inhibited JH synthesis by the corpora allata and JH synthesis was resumed by the corpora allata after oviposition (Readio et al. 1988).

Thus, we can conclude that reproduction in haematophagous insects consists of different behavioral and physiological processes that are closely regulated and which function in concert.

CHAPTER 3

MATING COMPETENCE OF *LU*. *ANTHOPHORA* (ADDIS) AND CHANGES IN MORPHOLOGY OF MALE REPRODUCTIVE SYSTEM DUE TO AGE AND SUCCESSIVE MATINGS

Introduction

Earlier studies on the reproductive biology of male sand flies dealt with their mode of copulation and the anatomical aspects of mating (Sinton 1925; Christopher & Barraud 1926; Sinton 1932; Hertig 1949; Sherlock & Carnerio 1964). Some information was also available about their epigamic behavior and the time required to complete insemination (Johnson & Hertig 1961; Chaniotis 1967; Ward 1977; Endris 1982). Sherlock & Carnerio (1964) described similarities and differences in the morphology of the male reproductive system in some sand flies and discussed the importance of the male anatomy as a taxonomic tool.

Generally, however, more information has been obtained about the reproductive system of female haematophagous insects because of their role in the spread of disease. Studies of female reproduction have also received attention because of their control by hormones and the significant morphological changes observed in the eggs after blood feeding.

Recently, insecticide resistance has evolved in many haematophagous insects and has prompted a search for new insect control methods. One such method is to release sterile males that are produced by genetic manipulation and chemosterilization. Such a technique, if ever devised for sand fly control, would require a vast knowledge about their mating behavior in nature. In particular, a study of changes in the morphology of the male reproductive system caused by aging and mating would be helpful in assessing the mating competitiveness of laboratory reared males (Reisen et al. 1981).

The objectives of the present investigation were: 1) to investigate the epigamic behavior of male and female _Lu_. _anthophora_; 2) to study the effect of age on the mating competence of virgin females and males; 3) to study the morphology of the male reproductive system and 4) to evaluate changes in the male reproductive system caused by increasing age and multiple matings.

Materials and Methods

Strain. _Lu_. _anthophora_ (Addis), was originally colonized from Texas (Addis 1945a, b). The present strain was colonized from Texas 8 years ago (Endris et al. 1982).

Colony Maintenance. During the present study, the colony of _Lu_. _anthophora_ was reared in an environmental chamber (Hotpack model 434310), maintained at 26 ± 2°C, 80-

85% relative humidity and with a 16:8 L:D photoperiod. The rearing procedure followed Young et al. (1981) and Endris et al. (1982).

Adults were maintained in 12x25.5x21.5 cm glass aquarium cages lined with plaster of Paris on three sides (Endris et al. 1982). Adults were provided with 10% sucrose solution on cotton pledgets as well as fresh apple slices; the latter was replaced daily and the former was changed every third day. Young (3-day-old) females were blood fed on an anesthetized hamster, immobilized with ketamine hydrochloride (Ketaset^R) that was injected intramuscularly (0.2 ml/adult hamster). Females were transferred 24 hrs after blood feeding to 120 ml oviposition tubes, covered at the bottom with plaster of Paris. Sugar solution was constantly available to these females. Females oviposited 4 to 6-days after the blood meal and the eggs hatched 8 days following oviposition.

Larvae were furnished with an aged 1:1 mixture (by volume) of dry rabbit feces and Purina rabbit chow (complete diet 5315^R) as food (Young et al. 1981). Larvae were reared in 120 ml containers, lined with plaster of Paris. A maximum of 100 larvae was placed in each container and ample food, along with a few drops of water, was added every other day to avoid competition. Pupae were placed in another container one day after their formation.

Investigation of Epigamic Behavior

The behavioral patterns of males and females before, during, and after mating were determined as follows. Ten 3 to 5-day-old virgin males were allowed to cohabit with 10 virgin females of the same age in an ice cream carton (1 pint) lined with plaster of Paris at the base and covered on top with a fine mesh screen. The mating behavior was observed for 3-hrs. The time required for complete insemination of a female by a male was determined by allowing one 5-day-old virgin male to cohabit with five virgin females of the same age and noting the time required for complete insemination. The observations were repeated 5 times. The epigamic behavior of both sexes, before and during initiation of mating, was also noted. Similarly, variable numbers of males and females were isolated in an aquarium cage and the different epigamic behaviors of both sexes observed before, during and after blood feeding.

The Effect of Age on Mating Competence

The effect of age on the mating competence of virgin females and males of different ages was determined as follows.

Females. Female pupae were sexed by checking the last abdominal segment then isolated for emergence in separate vials. After emergence, 250 females were kept in the aquarium cages and were aged for 0, 1, 2, 3, and 4 days. Similarly, 250 male pupae were sexed by examining their last abdominal segment and the males were aged after emergence in aquarium cages.

Four to five groups, each of 10 females, of each of the above ages were allowed to cohabit in 1 pint ice cream carton cages with 4-day-old males for one night (1800-0900 hrs). The following day, the spermathecae of all females were dissected in normal saline (0.9 g NaCl/litre) and the number of inseminated females was recorded. The amount of sperm and male accessory gland substance (MAGS) transferred by the males to the spermathecae of females was visually observed.

Virgin males. Two hundred male pupae were allowed to emerge in an aquarium cage. Males with terminalia rotated 0°-45° were isolated into separate cages and provided 10% sucrose solution and fresh apple slices as sugar. The males were aged for 0, 1, 2, 3, 5 and 7 days. Single males of the above ages were allowed females were allowed to cohabit in 1 pint ice cream cartons with 5 virgin 2 to 3-day-old females overnight. The following morning, the spermathecae of all females were dissected and number of inseminated females recorded. Males were saved for later dissections to determine the effect of age and mating on their reproductive system.

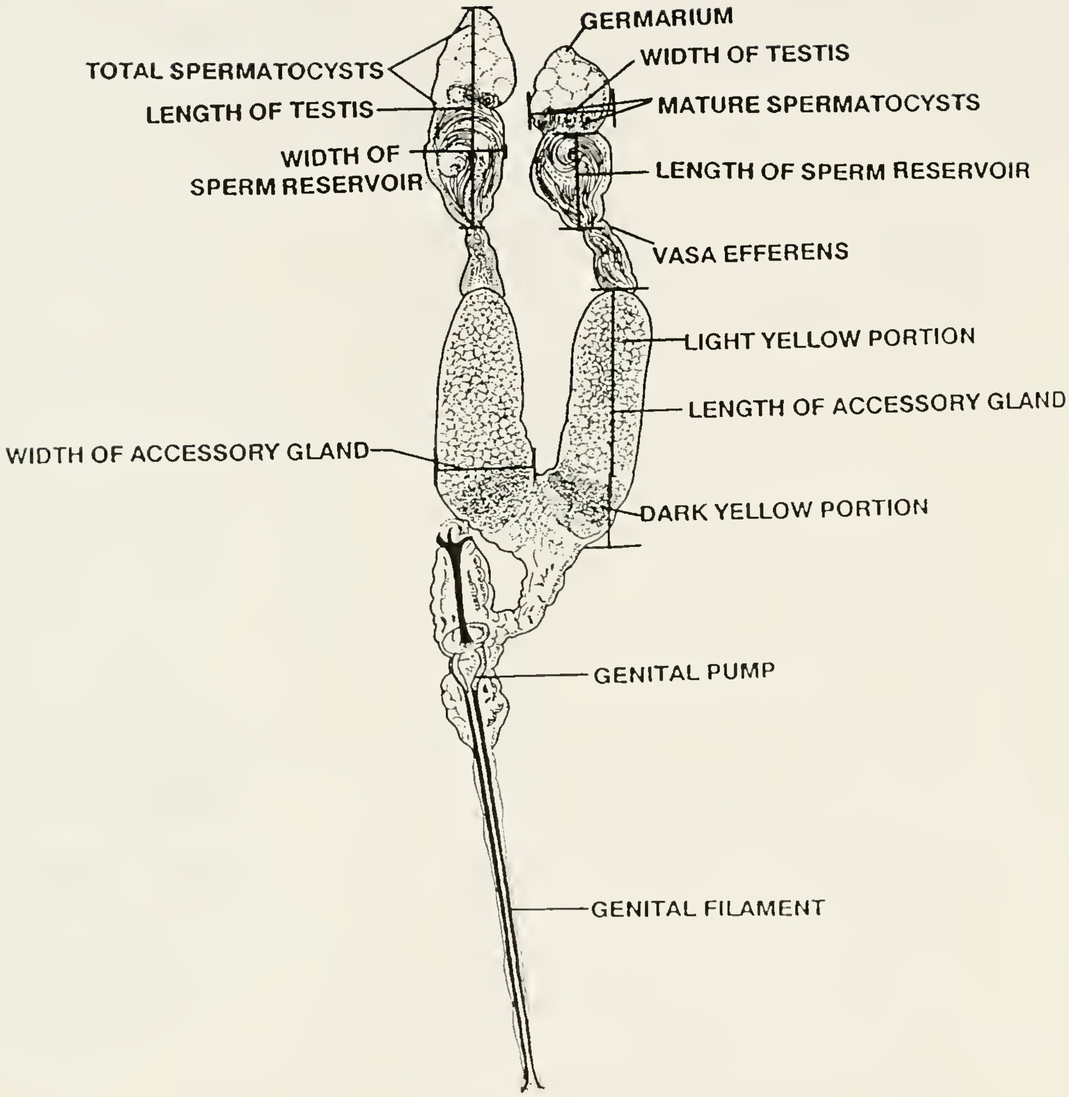

Figure 3-1. The reproductive system of male Lutzomyia anthophora. Distance between brackets represents the parts of structures measured.

Effects of Age and Mating on the Morphology of Male Reproductive System

The effect of age on the reproductive system of virgin males was investigated as follows. Two hundred male pupae were separated and the males were allowed to emerge. Two hundred individuals with terminallia rotated 0°-45° were isolated in an aquarium cage and allowed to age for 0, 1, 2, 3, 5 and 7 days. At each age, 10 males were dissected; different parts of their reproductive system were measured with a calibrated ocular micrometer under a compound microscope (Fig. 3-1).

To observe the reproductive system in mated males, individuals of different ages (0 to 7-days) and matings (1 to 5) were obtained from the mating experiment already discussed. These insects were dissected by the following method and different parts of their reproductive system were measured (Fig. 3-1).

Dissections and measurements. A single live male was blown from a mouth aspirator into a petri dish containing dilute detergent solution (dish washing soap). This procedure removed the majority of scales from the male's body. The male was immediately transferred into a drop of normal saline and washed several times to remove the detergent. Later, the male was transferred to another drop of normal saline on a glass slide and dissected with fine dissection needles under a microscope at 30 X. One needle was placed on the last abdominal segment and the other on the thorax, allowing excision of the whole reproductive system by traction on the last abdominal segment.

The following parts (Fig. 3-1) of the male reproductive system were measured without applying a cover slip (Mahmood & Reisen 1982; Mahmood et al. 1986): 1) length and width of testes; 2) length of portion containing the spermatocyst; 3) length and width of sperm reservoir; 4) total number of spermatocysts; 5) number of mature spermatocysts (spermatocysts containing spermatozoa); 6) length and width of accessory glands; 7) Width of seminal vesicles. Color and the degree of repleteness of the accessory glands with MAGS and sperms (if any) were also noted. Similarly, the amount of sperm in the sperm reservoir and seminal vesicles was also recorded photographically on a Zeiss microscope.

Results

Mating Behavior of Sand Flies

Virgin 5-day-old males initiated their epigamic behavior within 0-10 minutes after being placed in the cages containing the virgin females. The epigamic behavior of the two sexes consisted of the following steps.

1) The males reached the females following a zig zag path, during which time they vigorously flapped their wings

and moved their last abdominal segment up and down and sometimes sideways.

2) After reaching the female, most of the males either faced the females or were parallel to her, facing in the same direction.

3) When in contact, the males turned 180° angle and coupled tip to tip with the female their heads facing in the opposite directions. Not all attempts made by the males were successful. Sometimes the female disengaged herself and flew away within seconds.

4) Some mating attempts lasted from a few seconds to 4 to 5-minutes. A successful mating was achieved when the it lasted at least more than 10 minutes. After a successful mating the female disengaged herself. The males continued to flap their wings during copulation.

5) Many times virgin females moved near the mating couple but only as close as 1-5 cm. The presence of another female did not distract the mating couple.

6) The male attempted to copulate with a new female within the first five minutes after the completion of first mating. One male started to mate for the first time 11 minutes after it was placed in a cage with virgin females. The first mating lasted for 18 minutes. Seven minutes after the first mating, the male commenced following new females and attempted to mate with at least four different females. Although these females disengaged themselves within few

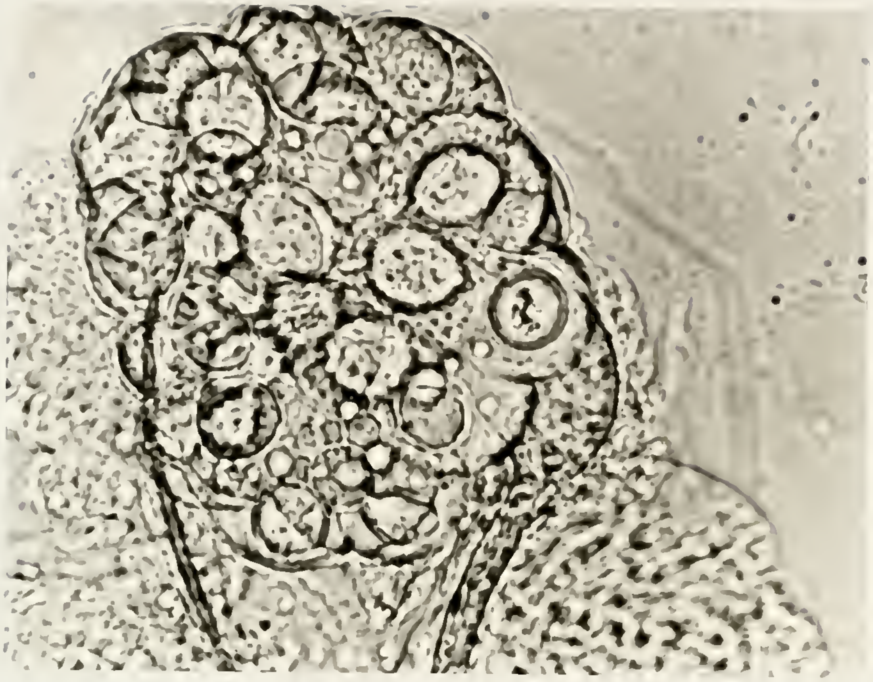

Figure 3-2. Spermathecae of a mated Lu. anthophora.

seconds. The male's second successful mating started 15 minutes after the first mating and lasted 12 minutes. His third mating started 41 minutes after the second and the couple remained together for 22.5 minutes. This male was dissected immediately (after the 3rd mating) and was found to have a very small sperm reservoir and a small amount of accessory gland substance.

The female sand flies were also dissected and spermathecae were checked for the presence of accessory gland substance and spermatozoa. The female has two elongated spermathecae that are surrounded by bubbles or evaginations at the tip of the elongated portion (Fig.3-2). The spermathecae open into two lateral spermathecal ducts that join into a common spermathecal duct. The first female that mated with the above discussed male showed a very dark yellow colored spermathecae. The yellow color was imparted by the male accessory gland substance (MAGS) that accumulated in the bubble like evaginations which became larger. Spermatozoa, mixed with accessory gland substance, were present in the spermathecae and the two spermathecal ducts. A reduced amount of MAGS was present in the spermathecae of 2nd and 3rd mated females compared to the amount found in the spermathecae of the first female. The amount of sperm increased in the second and third female. One male that mated with 5 females, transferred only spermatozoa and a few yellow accessory gland granules to the last female.

The males competed for females in cages where 10 males and 10 females were added together. Two to 3 males followed the same female and all of them tried to mate with the same female but only one male succeeded. At certain times, no males that followed the female were able to mate. Certain males tried to mate with a female that was already in copula with another male. Such males tried unsuccessfully to push their termanillia at right angle to the mating couple.

One female mated with 5 other males within a 40 minute period, although each mating lasted from few seconds to 4.5 minutes. The length of the first matings varied; for example, 3, 5, 13.5, 17.5, 18, 19, 35, and 38 minutes were observed for different males.

In the colony aquaria, males mated frequently with blood fed females just after blood feeding and sometimes with females one day after they had blood fed.

Effect of Age on the Mating Competence of Lu. anthophora

Females. No significant difference was present in the mating ability of 0 to 4-day-old females when compared by analysis of variance ($P > 0.05$). Maximum insemination percent (Mean ± S.E.M)(87 ± 3) were observed in one day old females. which were capable of mating a few hrs after emergence and later showed the following mating percentages.

Table 3-1. Effects of age on the mating ability of virgin males of _Lu_. _anthophora_.

		No. Females inseminated*					Male**	Female**
Age (Days)	No. Males	1	2	3	4	5	mated/total	mated/total
2	20	10	10	40	20	15	0.95	0.67
3	26	4	46	31	11		0.92	0.45
5	29	14	28	17	14	14	0.87	0.49
7	23	9	26	26	22	4	0.87	0.50

* Percentage of individual mated males when they were offered 5 virgin 2 to 3-day-old females for one night.

** Proportion of mated males or females.

Newly-emerged to < 24-hr-old females (71 ± 6), 2-day-old (75 ± 4), 3-day-old (77 ± 7) and 4-day-old (69 ± 7) percent females were inseminated during 12-hrs of cohabitation.

Virgin males. Males became sexually mature 13 to 24-hrs after emergence. The maximum number of females (67 %) were inseminated by 24 to 48-hr-old males (Table 3-1). There was no significant difference in the mean number of females inseminated per male ($P > 0.05$). The maximum mean number (3.1 females per male) was inseminated by 24 to 48-hr-old males. Three-day-old males inseminated 2.4 females per male and 2.5 females per male were inseminated by 5 to 7-day-old males. Each male was capable of inseminating 0-5 females under the present conditions. Mating ability of males was restricted due to the presence of only 5 females. The maximum number of 5-day-old males did not mate during 12 hrs of cohabitation. Maximum percent of 24 to 48-hr-old males were capable of inseminating 3-5 females (Table 3-1). Maximum number of 3-day old males inseminated 2 females and no male mated with 5 females in this group (Table 3-1).

Morphology of the Male Reproductive System

The male reproductive system consist of a pair of oblong testes each of which has a variable number of round ball-shaped spermatocysts. Testes of both sides opened into separate vasa efferentia that became dilated at their lower end to form the seminal vesicles which store sperms. No

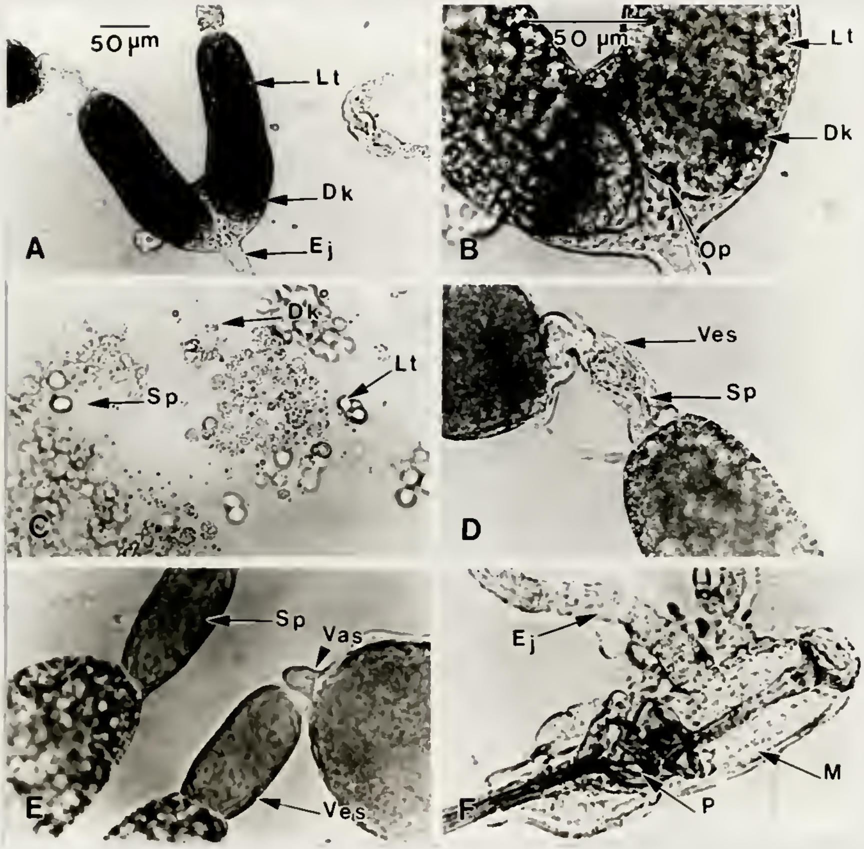

Figure 3-3. Different parts of the reproductive system of Lutzomyia anthophora. A = accessory glands of newly emerged male; B = posterior portion of the accessory glands of newly emerged male showing two types of accessory gland secretions; C = male accessory gland secretions showing light yellow granules (Lt), dark yellow granules (Dk) and some sperms are also visible.; D = seminal vesicles (Ves) from newly emerged male showing small amount of spermatozoa (Sp); E = seminal vesicles of 2-day-old virgin male showing large amount of spermatozoa. The vasa efferens is also visible; F = genital pump showing the ejaculatory duct (Ej), muscular sheath (M) and chitinous pump (P).

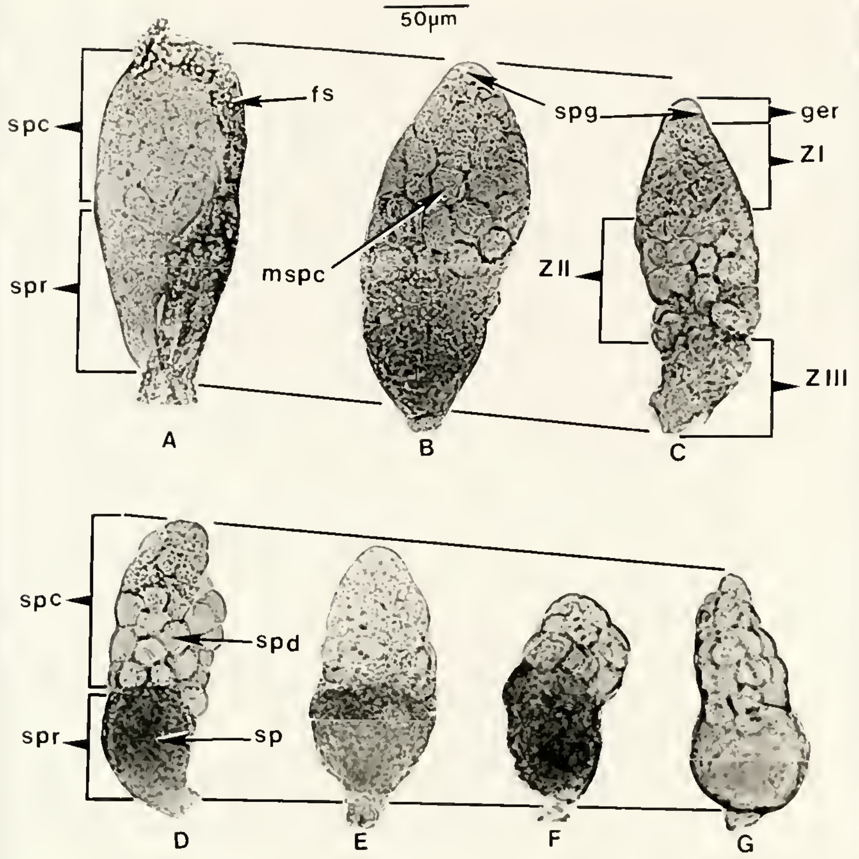

Figure 3-4. Effects of age on the testis of virgin *Lu. anthophora*. A = testis of mature pupae showing sperm reservoir (spr), spermatocyst containing portion (spc) and fat sheath around the testis; B = testis of a newly emerged male (Terminalia 1/2 rotated) showing mature spermatocyst (mspc) and spermatogonia (Spg); C = testis of 1-day-old male showing germarium (ger), zone of growth (ZI), zone of maturation and reduction (ZII) and zone of transformation (ZIII); D = testis of 3-day-old male showing spermatids (spd) and spermatozoa (sp) in the sperm reservoir; E = testis of 4-day-old male; F = testis of 5-day-old male and G = testis of 7-day-old male. Note the progressive decrease in the size of testis, number of total and mature spermatocysts.

vasa deferentia are present. The seminal vesicle on each side opens into separate oblong lobe of the accessory gland that joins posteriorly into a common cup-shaped portion. The common portion of the accessory glands opens into an ejaculatory duct (Figs. 3-1 & 3-3-A, 3-3-B & 3-3-C).

The ejaculatory duct is a thin tube with muscular walls that opens into the genital pump. The genital pump is joined by two long genital filaments (Figs. 3-1 & 3-3-F). Both the genital pump and genital filaments are encased in a muscular sheath (Fig. 3-1).

Each lobe of the accessory gland has two distinct regions, an anterior portion filled with large light yellow granules (Lt) and a small dark yellow posterior band with cells secreting small dark yellow granules (Figs. 3-1 & 3-3-A,3-3-B & 3-3-C). Each accessory gland has an opening (op) or canal in the posterior band-shaped area. During mating this portion strongly contracts to pump the seminal fluid into the ejaculatory duct.

The testis on each side is enveloped by a soft sheath of fat bodies (fs) and is attached to the body wall by a soft filament (Fig. 3-4-A). Germarium, composed of few actively dividing spermatogonia, is present at the tip of each testis. Active cell division occurs in zone I (ZI) (zone of growth) and immature spermatocysts containing fat fusiform spermatozoa are found here. Each spermatocyst is derived from a single sperm mother cell. Zone II (ZII) (or

Table 3-2. Effects of age on the morphology of the male reproductive system of virgin Lu. anthophora.

	Age (Days) $(\bar{X} \pm SE\bar{X})^*$				
Characteristics	0	2	3	5	7
Testes					
Length (µm)	180 ± 0^{a}	169 ± 0^{ab}	165 ± 10^{b}	145 ± 0^{c}	138 ± 10^{c}
Width (µm)	68 ± 0^{a}	67 ± 0^{a}	57 ± 0^{b}	49 ± 0^{c}	46 ± 0^{c}
Spermatocyst portion					
Length (µm)	136 ± 30^{a}	93 ± 10^{b}	90 ± 10^{b}	66 ± 0^{b}	53 ± 0^{b}
Sperm reservoir					
Length (µm)	83 ± 0^{a}	80 ± 0^{ab}	78 ± 0^{ab}	73 ± 0^{ab}	69 ± 0^{b}
Width (µm)	62 ± 0^{a}	59 ± 0^{a}	57 ± 0^{a}	58 ± 0^{a}	58 ± 0^{a}
Total spermatocysts	22 ± 1^{a}	18 ± 1^{b}	13 ± 1^{c}	11 ± 1^{d}	9 ± 1^{e}
Mature spermatocysts	12 ± 1^{a}	11 ± 1^{a}	4 ± 1^{b}	4 ± 1^{b}	3 ± 0^{b}
Accessory glands					
Length (µm)	210 ± 0^{b}	222 ± 0^{a}	216 ± 0^{a}	222 ± 0^{a}	215 ± 0^{a}
Width (µm)	56 ± 0^{ab}	58 ± 0^{a}	57 ± 0^{ab}	54 ± 0^{b}	56 ± 0^{ab}
Seminal vesicle					
Width (µm)	NE**	27 ± 0^{a}	NE**	25 ± 0^{a}	26 ± 0^{a}

* Mean ± Standard error of the mean, followed by same letters in each row were not significantly different ($P > 0.05$) when compared by Duncan's new multiple range test (Lyman Ott 1988). ** Not estimated.

zone of maturation) has mature spermatocysts (or spermatocysts with differentiated spermatozoa). Spermatozoa are released from ZII to Zone III (ZIII) (or zone of transformation) and remain here to form the sperm reservoir (Fig. 3-4-C).

After rupture of the spermatocyst wall, spermatozoa from a single spermatocyst remain together and descend in the form of sperm balls that accumulate in the sperm reservoir (Figs. 3-1 & 3-4). The mature spermatozoa descend from the sperm reservoir into the vasa efferens and accumulate into the seminal vesicles.

Effects of Age and Mating on the Morphology of the Reproductive System

Virgin males

Motile spermatozoa were present in the sperm reservoir and seminal vesicles and accessory glands of newly emerged males were filled with MAGS. The following changes occurred in the reproductive system of 0, 1, 2, 3, 5 and 7-day-old males due to age.

Testes. Testes became significantly smaller and narrower in older males, being longest and widest in newly-emerged males and shortest and narrowest in 7-day-old males ($P < 0.05$) (Table 3-2)(Fig. 3-4). Peak spermatogenesis occurred in 0 to 2-day-old males, because maximum number of mature spermatocysts were present at this age (Table 3-2).

The mean number of total and mature spermatocysts significantly decreased with increasing age ($P < 0.05$)(Table 3-2) (Fig. 3-4). This resulted in a significant decrease in the length of the spermatocyst containing portion ($P < 0.05$).

No significant effect of aging was observed on the length and width of sperm reservoir ($P > 0.05$). The amount of accumulated sperm increased in the sperm reservoir of older males (Fig. 3-4).

Accessory glands. The amount of MAGS increased in older males. The accessory glands of such males appeared dark yellow under a dissecting microscope (Fig. 3-3-A, 3-3-B). Increase in MAGS resulted in a nonsignificant increase in the size of the accessory glands ($P > 0.05$). Smallest accessory glands were present in newly emerged males and 2-day-old males had the largest glands (Table 3-2). Occasionally, a few sperm were seen mixed with the pale yellow accessory gland granules (Fig. 3-3-C).

Seminal vesicles. The width of the seminal vesicles which contained small amount of sperms was not estimated in newly emerged males (Fig. 3-3-D). No significant variation occurred in the width of the seminal vesicle of older males; they were packed with actively moving spermatozoa ($P > 0.05$)(Fig. 3-3-E)(Table 3-2).

Mated males

The effects of age and mating were assessed by the significance level of age-by-mating interaction term in the analysis of variance.

Testes. The lengths of testes significantly decreased due to age and mating ($P < 0.05$). Virgin males possessed the longest testes. The length decreased gradually in 1-5 mated males. The shortest testes were seen in 5-mated males. The width of testes also decreased due to age ($P < 0.05$) and no age-by-mating interaction was present ($P > 0.05$). The testes of 2-day-old males were significantly wider than 3, to 7-day-old males (Table 3-2).

Both age and matings played a significant role in decreasing the length of the spermatocyst-containing portion ($P < 0.05$). Five-mated males had the longest spermatocyst-containing portion but it was not significantly different from 1 to 4-mated males. Two-day-old males had the longest spermatocyst-containing portion compared to 3 to 7-day-old males that showed a gradual decrease with increasing age.

There was no effect of matings on the mean number of spermatocysts, but the effect of age was significant ($P < 0.05$). Thus, the age-by-mating interaction was nonsignificant ($P > 0.05$). The number of mature spermatocysts was affected by both age and mating, since age-by-mating interaction was significant ($P < 0.05$). Two-day-old males had a maximum number of mature spermatocysts and the number gradually declined in 3 to 7-day-old males.

The length of the sperm reservoir significantly shortened due to the effects of both age and matings ($P < 0.05$). Virgin males of all ages have a longer sperm reservoir that was not significantly different from 2-mated males. There was no difference in the length of sperm reservoir of 1 to 3-mated males. The 4 to 5-mated males had the shortest sperm reservoir.

No interaction was present between age and mating for the width of sperm reservoir ($P > 0.05$), but when considered separately, the effect of mating was highly significant ($P < 0.05$). Virgin males had a wider sperm reservoir than that of 1 to 5-mated males. The narrowest width of the sperm reservoir was seen in 5-mated males but it was not significantly different from 3 to 4-mated males. Similarly, 1 to 2-mated males were not different from each other. The width of the sperm reservoir was not significantly different between 2 to 7-day-old males ($P > 0.05$).

Accessory glands. Both the length and width of accessory glands decreased significantly due to age and matings ($P < 0.05$). The Longest and widest accessory glands were present in virgin males when compared to 1-5-mated males. The length of accessory glands was not different in 1 to 2-mated males or of 2 to 4-mated. Once-mated males had

Table 3-3. Effects of matings on the morphology of the male reproductive system of Lu. *anthophora*.

Characteristics* (Matings)	Age (Days) 2	3	5	7
Testes length ($\overline{\mu m}$ ± SE$\overline{X}$)				
0	169 ± 4 (20)^{ab}	162 ± 10 (20)^{a}	145 ± 0 (20)^{a}	138 ± 10 (20)^{ab}
1	138 ± 7 (4)^{cd}	140 ± 10 (4)^{a}	139 ± 10 (8)^{a}	158 ± 20 (2)^{a}
2	184 ± 0 (0)^{a}	142 ± 10 (14)^{a}	136 ± 10 (10)^{a}	131 ± 10 (10)^{abc}
3	127 ± 6 (6)^{cd}	149 ± 10 (9)^{a}	122 ± 10 (9)^{a}	128 ± 0 (18)^{bc}
4	147 ± 7 (8)^{bc}	126 ± 20 (3)^{a}	129 ± 10 (9)^{a}	112 ± 10 (10)^{bc}
5	112 ± 0 (2)^{d}		124 ± 10 (10)^{a}	107 ± 10 (2)^{c}
Testes width ($\overline{\mu m}$ ± SE$\overline{X}$)				
0	67 ± 2 (20)^{a}	57 ± 0 (20)^{a}	47 ± 0 (20)^{a}	49 ± 0 (20)^{a}
1	59 ± 3 (4)^{a}	51 ± 0 (4)^{a}	51 ± 0 (8)^{a}	51 ± 0 (2)^{a}
2	51 ± 0 (2)^{a}	52 ± 0 (14)^{a}	52 ± 0 (10)^{a}	49 ± 0 (14)^{a}
3	62 ± 7 (9)^{a}	57 ± 10 (9)^{a}	51 ± 0 (9)^{a}	45 ± 0 (14)^{a}
4	54 ± 2 (8)^{a}	58 ± 0 (3)^{a}	50 ± 0 (9)^{a}	48 ± 0 (10)^{a}
5	56 ± 5 (2)^{a}		47 ± 0 (8)^{a}	41 ± 10 (2)^{a}
Length of spermatocyst portion ($\overline{\mu m}$ ± SE$\overline{X}$)				
0	98 ± 40 (20)^{a}	90 ± 10 (20)^{a}	66 ± 0 (20)^{a}	53 ± 0 (20)^{a}
1	84 ± 88 (4)^{a}	69 ± 0 (4)^{a}	67 ± 10 (8)^{a}	61 ± 10 (2)^{a}
2	97 ± 50 (2)^{a}	69 ± 10 (11)^{a}	64 ± 0 (10)^{a}	62 ± 0 (14)^{a}
3	81 ± 65 (10)^{a}	82 ± 10 (8)^{a}	55 ± 10 (9)^{a}	63 ± 0 (18)^{a}
4	79 ± 83 (7)^{a}	82 ± 10 (8)^{a}	70 ± 10 (9)^{a}	58 ± 0 (10)^{a}
5	72 ± 11 (2)^{a}		62 ± 10 (8)^{a}	66 ± 10 (2)^{a}

Table 3-3--extended.

Characteristics* (Matings)	Age (Days) 2	3	5	7
Length of sperm reservoir $(\overline{\mu m} \pm \overline{S}EX)$				
0	73 ± 30 (20)^{ab}	80 ± 0 (20)^{a}	78 ± 0 (20)^{a}	83 ± 0 (20)^{ab}
1	54 ± 30 (4)^{cd}	69 ± 0 (4)^{a}	68 ± 10 (7)^{ab}	97 ± 10 (2)^{a}
2	87 ± 50 (2)^{a}	73 ± 10 (12)^{a}	74 ± 0 (9)^{ab}	71 ± 10 (14)^{bc}
3	45 ± 30 (10)^{d}	69 ± 10 (9)^{a}	67 ± 10 (9)^{ab}	65 ± 0 (18)^{bc}
4	67 ± 30 (8)^{bc}	34 ± 10 (3)^{b}	59 ± 0 (9)^{b}	54 ± 0 (10)^{cd}
5	41 ± 10 (2)^{d}		67 ± 10 (8)^{ab}	41 ± 10 (2)^{d}
Width of sperm reservoir $(\overline{\mu m} \pm SE\overline{X})$				
0	59 ± 2 (20)^{a}	62 ± 0 (20)^{a}	57 ± 0 (20)^{a}	58 ± 0 (20)^{a}
1	48 ± 5 (4)^{abc}	48 ± 0 (4)^{b}	53 ± 0 (8)^{ab}	51 ± 0 (2)^{ab}
2	56 ± 5 (2)^{ab}	44 ± 0 (14)^{b}	53 ± 0 (10)^{ab}	45 ± 0 (14)^{b}
3	37 ± 3 (9)^{c}	48 ± 0 (9)^{b}	45 ± 0 (9)^{bc}	44 ± 0 (16)^{b}
4	43 ± 2 (8)^{bc}	34 ± 0 (3)^{c}	43 ± 0 (9)^{c}	40 ± 0 (10)^{b}
5	36 ± 5 (2)^{c}		42 ± 0 (8)^{c}	41 ± 0 (2)^{b}
Total spermatocysts $(\overline{No.} \pm SE\overline{X})$				
0	18 ± 1 (20)^{a}	13 ± 1 (20)^{b}	11 ± 1 (20)^{a}	9 ± 1 (20)^{a}
1	18 ± 2 (4)^{a}	16 ± 1 (4)^{ab}	11 ± 1 (8)^{a}	10 ± 1 (2)^{a}
2	20 ± 0 (3)^{a}	13 ± 1 (14)^{b}	10 ± 1 (10)^{a}	9 ± 0 (14)^{a}
3	19 ± 1 (7)^{a}	15 ± 1 (9)^{ab}	10 ± 1 (9)^{a}	9 ± 0 (16)^{a}
4	15 ± 2 (8)^{a}	18 ± 2 (5)^{a}	12 ± 1 (9)^{a}	10 ± 1 (10)^{a}
5	19 ± 1 (4)^{a}		11 ± 1 (8)^{a}	9 ± 1 (2)^{a}

Table 3-3--continued.

Characteristics* (Matings)	Age (Days)			
	2	3	5	7
Mature spermatocysts $(\overline{\text{No.}} \pm \text{SE}\overline{\text{X}})$				
0	12 ± 1 (16)^{a}	4 ± 1 (20)^{a}	3 ± 0 (20)^{a}	4 ± 1 (20)^{a}
1	9 ± 1 (4)^{ab}	8 ± 1 (4)^{a}	4 ± 2 (6)^{a}	4 ± 0 (2)^{a}
2	8 ± 0 (2)^{ab}	7 ± 1 (14)^{a}	3 ± 1 (9)^{a}	3 ± 0 (14)^{a}
3	4 ± 2 (7)^{b}	7 ± 1 (9)^{a}	4 ± 1 (8)^{a}	4 ± 0 (16)^{a}
4	10 ± 1 (8)^{a}	6 ± 3 (3)^{a}	5 ± 1 (9)^{a}	3 ± 1 (10)^{a}
5	10 ± 2 (4)^{a}		6 ± 1 (8)^{a}	0 ± 0 (2)^{b}
Length of accessory glands $(\overline{\mu\text{m}} \pm \text{SE}\overline{\text{X}})$				
0	222 ± 20 (20)^{a}	216 ± 0 (20)^{ab}	222 ± 3 (20)^{a}	215 ± 10 (20)^{a}
1	181 ± 90 (4)^{b}	222 ± 0 (4)^{a}	192 ± 4 (8)^{bc}	204 ± 0 (2)^{a}
2	178 ± 50 (2)^{b}	199 ± 0 (16)^{b}	198 ± 6 (12)^{b}	181 ± 0 (14)^{b}
3	195 ± 20 (13)^{b}	177 ± 10 (4)^{c}	190 ± 6 (10)^{bc}	182 ± 0 (18)^{b}
4	187 ± 6 (8)^{a}	196 ± 20 (4)^{bc}	189 ± 7 (10)^{bc}	177 ± 0 (10)^{b}
5	194 ± 6 (4)^{b}		176 ± 5 (8)^{c}	163 ± 0 (2)^{b}
Width of accessory glands $(\overline{\mu\text{m}} \pm \text{SE}\overline{\text{X}})$				
0	58 ± 1 (20)^{a}	57 ± 0 (20)^{a}	54 ± 0 (20)^{a}	56 ± 0 (20)^{a}
1	41 ± 0 (4)^{c}	53 ± 0 (4)^{a}	50 ± 0 (8)^{ab}	56 ± 10 (2)^{a}
2	51 ± 0 (2)^{b}	46 ± 0 (16)^{b}	48 ± 0 (12)^{b}	45 ± 0 (14)^{b}
3	42 ± 1 (12)^{c}	41 ± 0 (10)^{b}	39 ± 0 (10)^{c}	43 ± 0 (18)^{b}
4	45 ± 2 (8)^{c}	41 ± 00 (2)^{b}	46 ± 0 (10)^{bc}	42 ± 0 (10)^{b}
5	46 ± 3 (4)^{bc}		39 ± 0 (8)^{c}	61 ± 0 (2)^{a}

Table 3-3--extended.

Characteristics* (Matings)	Age (Days) 2	3	5	7
Width of seminal vesicles ($\overline{\mu m} \pm SE\overline{X}$)				
0	27 ± 0 (20)^{a}	NE**	25 ± 0 (10)^{a}	26 ± 0 (10)^{a}
1	25 ± 0 (4)^{ab}	31 ± 0 (4)^{a}	24 ± 0 (8)^{a}	20 ± 0 (2)^{ab}
2	25 ± 0 (2)^{ab}	25 ± 0 (17)^{ab}	24 ± 0 (8)^{a}	22 ± 0 (14)^{ab}
3	18 ± 0 (12)^{c}	17 ± 0 (3)^{c}	25 ± 0 (10)^{a}	24 ± 0 (18)^{ab}
4	24 ± 0 (8)^{abc}	20 ± 0 (4)^{bc}	22 ± 0 (10)^{ab}	18 ± 0 (10)^{b}
5	20 ± 0 (4)^{bc}		18 ± 0 (8)^{b}	20 ± 0 (2)^{ab}

* Mean ± (standard error of the mean)(sample size) in each coloumn followed by the same letters were not significantly different ($P > 0.05$) when compared by Duncan's new multiple range test (Lyman Ott 1988). ** Not estimated.

significantly wider glands when compared to 2 to 5-mated males. The shortest length and narrowest width were observed in 5-mated males. There was no difference in the width of the accessory glands among 2, 3 and 7-days-old males.

Seminal vesicles. Age and matings both affected the width of the seminal vesicles ($P < 0.05$). There was no difference between the width of the seminal vesicle of virgin and 1-mated males. The width of the seminal vesicles was not different between 1 to 4-mated males but it was significantly larger in comparison to 5-mated males.

The effect of mating on different parts of reproductive system for each age was compared separately by Duncan's new multiple range test. The following changes were observed.

Two-day-old males. The length of the testis and length and width of the sperm reservoir varied significantly in virgin and mated males ($P < 0.05$). Two-mated males had the longest testes but were not different from 1-mated males ($P < 0.05$). Similarly, the length of the testes was not different between 1, 3 and 4-mated males ($P < 0.05$). Males that mated 5-times during a 12-hrs cohabitation period had the shortest testes (Table 3-3).

The length and width of the sperm reservoir decreased significantly following multiple matings. Virgin and 2-mated males showed no difference in the length of sperm reservoir; which were longer when compared to 1, and 3 to 5-

mated males ($P < 0.05$)(Table 3-3). Virgin males had the widest sperm reservoir but it was not significantly different from 1, 2 and 4-mated males. Therefore, the decrease in the total volume of sperm reservoir can be depicted either by decrease in length or width of sperm reservoir (Table 3-3). In 1 to 2-mated males the spermatozoa were utilized mostly from the seminal vesicles; whereas, in 3 to 5-mated males all spermatozoa from the seminal vesicles, and some from the sperm reservoir, were also transferred to the females. The sperm reservoir of 3 to 5- mated males were pale.

The length and width of the accessory glands and width of the seminal vesicles decreased in mated males ($P < 0.05$). The longest and widest sperm reservoir was observed in virgin males. Multiple matings had no effect on the length of accessory gland of 1 to 5-mated males but the width of accessory glands of 1, 3 and 4-mated males was smaller than virgin and 2-mated males ($P < 0.05$)(Table 3-3). The smallest width of the seminal vesicles was seen in 5-mated males which contained few sperm.

No difference was found in the length and width of the spermatocyst-containing portion and the mean number of the total spermatocysts ($P > 0.05$). The mean number of mature spermatocysts decreased significantly; 3-mated males had smallest number of mature spermatocysts ($P < 0.05$)(Table 3-3).

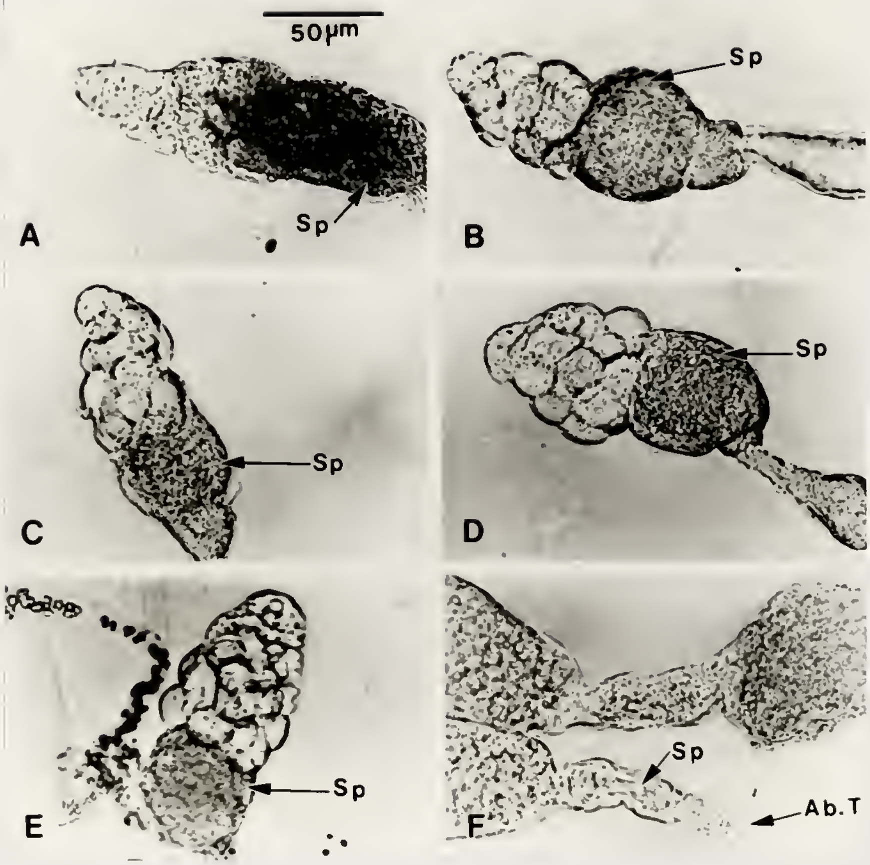

Figure 3-5. Effects of matings on the testes of 7-day-old Lu. anthophora showing progressive decrease in the size of sperm reservoir and amount of spermatozoa (Sp) after successive matings; A = testis of 1-mated male; B = testis of 2-mated male; C = testis of 3-mated male; D = testis of 4-mated male; E = testis of 5-mated male and F = an abnormal testis (Ab.T) of 7-day-old male.

Three-day-old males. No change due to mating was detected in the length of testes, length and width of spermatocyst-containing portion, and the mean number of total and mature spermatocysts ($P > 0.05$) (Table 3-3).

The sperm reservoir and accessory glands became shorter and narrower due to increased matings and the width of the seminal vesicles decreased with successive matings ($P < 0.05$) (Table 3-3). The smallest sperm reservoir that was light in color and contained small amount of sperms was of 4-mated males. The smallest accessory glands were observed in 3-mated males which were not significantly different from 4-mated males($P > 0.05$).

Five-day-old males. No significant change occurred due to mating in the length of testes, length and width of the spermatocyst-containing portion, mean number of total and mature spermatocysts and width of the seminal vesicles ($P > 0.05$) (Table 3-3). Successive matings resulted in a decrease in the length and width of the sperm reservoir and the accessory glands of 1 to 5-mated males ($P < 0.05$) (Table 3-3).

Seven-day-old males. No significant change occurred in the width of the testes, length of the spermatocyst-containing portion and mean number of total and mature spermatocysts due to successive matings ($P > 0.05$) (Table 3-3) (Fig. 3-5). Although 7-day-old, 5-mated males did not have any mature spermatocyst, no conclusion should be drawn

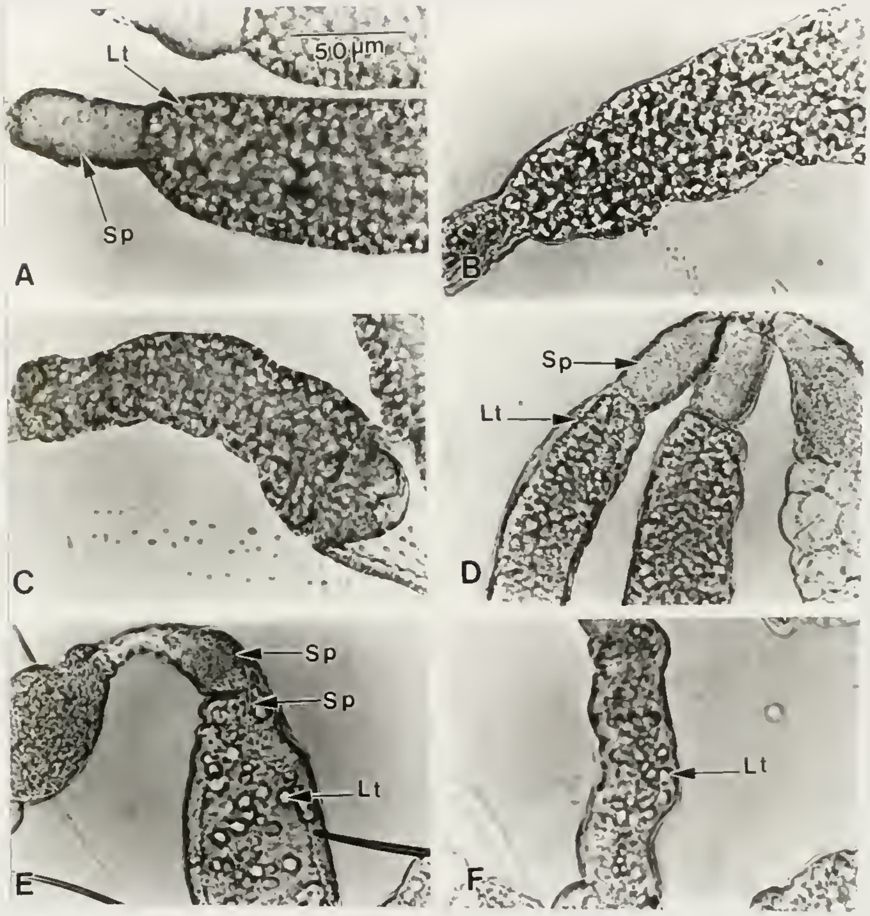

Figure 3-6. Effects of succesive matings on the anterior portion of the accessory glands and seminal vesicles of 7-day-old male Lu. anthophora, showing progressive decrease in size and depletion of Light yellow MAGS (Lt) and spermatozoa (Sp); A = accessory gland of virgin 7-day-old male showing large amount of light yellow MAGS (Lt) and the seminal vesicle replete with spermatozoa; B = once-mated male; C = twice-mated male showing decrease in size of accessory gland; D = three times mated male; E = few light yellow granules of MAGS in the accessory glands of 4-mated male; F = shrunken and depleted accessory gland of a 5-mated male.

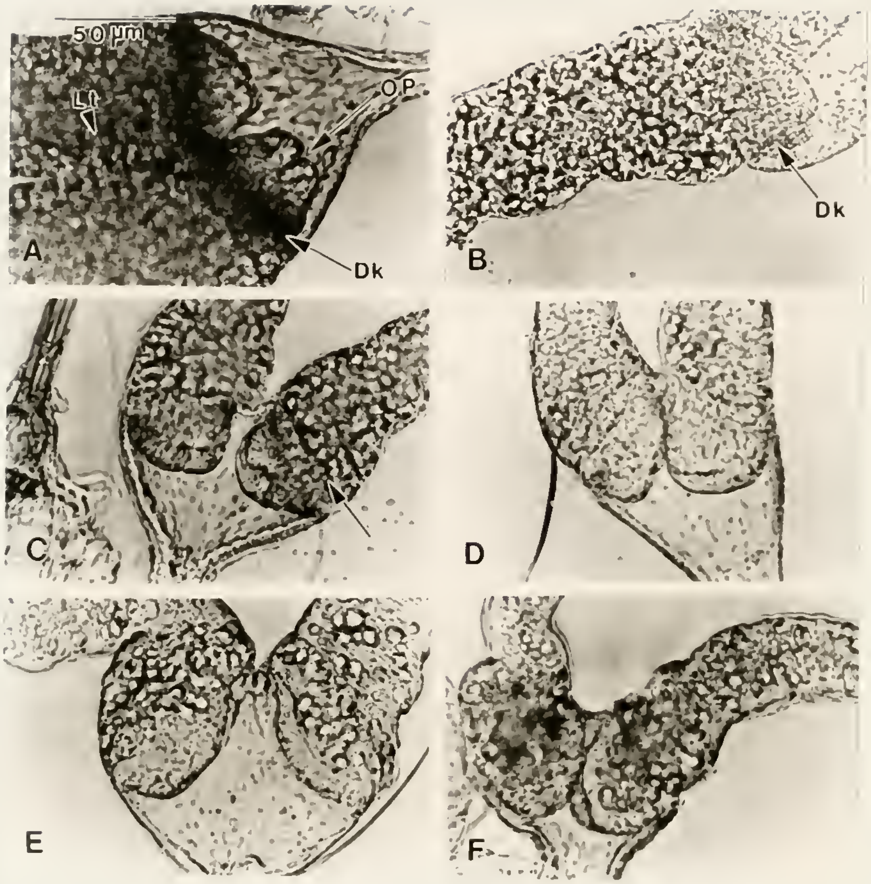

Figure 3-7. Effects of matings on the posterior portion of the accessory glands of 7-day-old male *Lu. anthophora* showing progressive decrease in size and depletion of dark yellow MAGS (Dk) after successive matings; A = accessory gland of virgin 7-day-old male showing large amount of dark yellow MAGS (Dk); B = once-mated male showing small amount of dark yellow MAGS; C = twice-mated male, arrow points to the area that secretes dark yellow MAGS; D = three times mated male showing complete depletion of dark yellow MAGS; E = few light yellow granules of MAGS in the accessory glands of 4-mated male; F = shrunken and depleted accessory gland of a 5-mated male.

due to a very small sample size. A decrease in the length of testes, length and width of sperm reservoir and accessory glands occurred due to increased matings ($P < 0.05$) (Table 3-3) (Figs. 3-5, 3-6 & 3-7).

The following account deals with the overall changes that were visually observed in the reproductive system of males. Seven days old males are taken as an example because of full reproductive maturity.

The amount of sperm decreased in the sperm reservoir of 4 to 5-mated males which was paler than that of 2-mated males (Fig. 3-5-A to -E). The accessory glands of virgin males were dark yellow but as the accessory gland material was ejaculated during successive matings they became empty. The accessory glands of 4 to 5-mated males had only few light yellow granules in the anterior portion of the glands (Fig. 3-6-E & F). The walls of the anterior portion of the glands contracted which resulted in a decrease in the width of the accessory glands (Fig. 3-6-F).

The amount of dark yellow MAGS also decreased with an increased number of matings. Once-mated males lost light yellow MAGS from the anterior part of the accessory glands and had small amount of posterior dark yellow MAGS (Figs. 3-6-B, 3-7-B). After two matings, dark yellow granules were absent and the cells of posterior portion were easily separated from the anterior portion (Fig. 3-7-C to F). Accessory glands of 3 to 5-mated males lost most of the light yellow MAGS from the anterior portion of the glands (Fig. 3-6) and were devoid of small dark yellow MAGS in the posterior region (Fig. 3-6-D & 3-6-F). Sometimes spermatozoa could be easily seen in the anterior portion of the accessory glands of 4 to 5-mated males (Fig. 3-6-E).

Discussion

Lu. anthophora males displayed an epigamic behavior similar to that of Lu. vexator occidentis (Fairchild & Hertig) (Chaniotis 1967). Both sexes of Lu. anthophora were capable of mating any time of the day or night and before or after blood feeding. Males were never observed copulating with a blood feeding female. This is in contrast to Lu. v. occidentis males that Chaniotis (1967) observed mating with females that were blood feeding. Time spent in copula ranged from few seconds to 38 minutes. Thus, females that were in copula with a male for more than ten minutes were considered having mated successfully. The longest time spent in copula by Lu. V. occidentis females was 12 minutes. Similarly, Lu. californica (Fairchild & Hertig) and Lu. stewarti (Mangabeira & Galindo) spent 12 and 9 + minutes respectively in copula. Thus, it appears that in phlebotmine sand flies a longer time is required for successful insemination of a female. In contrast, mosquitoes, such as Cx. tritaeniorhynchus, a successful mating may last less than 30 seconds from initial contact to completion (24.75 ± 1.11 sec) (Reisen et al. 1979b).

Females were ready to mate within 12 hrs after emergence, unlike mosquitoes where female receptivity is age related and is triggered by JH secretions from the corpora allata (Lea 1968). Female *An*. *stephensi* were inseminated within 8 hrs of emergence but the proportion of inseminated females was very low (Mahmood & Reisen 1982).

The mating competence of *Lu*. *anthophora* was not significantly different in females up to 4 days after emergence. Again, this behavior differed from mosquitoes like *An*. *culicifacies*, in which the maximum number of females mated to sexually mature males when they were 2 to 4-day-old (Reisen et al. 1979a). The early maturity of phlebotomine females may be adaptive due to their relatively short life spans.

Two types of adaptive behaviors during mating were exhibited by males and females. When only one virgin male was added to a cage containing 5 virgin females, females actively moved near the male. Similarly, some females moved within 1-5 cm of a copulating pair where they remained. Their presence did not distract the couple. Both of these behaviors could be advantageous to the mating success of the receptive females. In colony cages, males actively searched for females and up to three males could be seen following a single female. Some males tried to mate with a female which was already in copula with another male. Such males reached at right angle to the mating couple and tried to grasp the female terminalia but these attempts were always unsuccessful.

Similar to earlier observations (Chaniotis 1967), multiple insemination of females by more than one male was a common occurrence. One female mated with 5 different males during one hour. None of these matings lasted for more than 5 minutes and the first 4 attempts lasted less than one minute each. Whether semen can be transferred during such short encounters is not conclusive because males have long genital filaments that must be inserted through the spermathecal duct and into the spermathecae during copulation (Hertig 1949).

The reproductive system of Lu. anthophora was well advanced at emergence compared to the reproductive system of some mosquitoes (Hausermann & Nijhout 1975; Jones 1967; Mahmood & Reisen 1982; Mahmood et al. 1986). The sperm reservoir and seminal vesicles of newly emerged males contains ample spermatozoa at emergence. The spermatozoa showed rhythmic undulating movements and possessed a head and long tail. Males were capable of mating 13-hrs after emergence when their terminalia fully rotated to 180°. The rapid development of mature reproductive system must be advantageous to these short lived insects, the males of which can mate with newly-emerged receptive females in their short life span.

The part of the reproductive system, designated as accessory glands in the present study was incorrectly identified as seminal vesicles by earlier authors (Perfil'ev 1928; Sherlock & Carneiro 1964; Jobling 1987). In my opinion this structure is the accessory gland which exhibits similar morphology and changes due to mating to those observed in mosquitoes (Dapples et al. 1974; Adlakha et al. 1976b; Mahmood & Reisen 1982; Mahmood et al. 1986).

Maximum spermatogenesis was observed in 0 to 2-day-old males and the greatest number of differentiated spermatocysts that contained spermatozoa was found at this age. The size of the testes and the mean number of spermatocysts decreased with increasing age in a manner similar to those changes found in mosquitoes (Hausermann & Nijhout 1975; Jones 1967; Mahmood & Reisen 1982; Mahmood et al. 1986). The percentage of the testes occupied by the sperm reservoir and width of the seminal vesicles increased in older males. Thus, the sperm released from the mature spermatocyst was stored there. At no time were spermatozoa found in the accessory glands and ejaculatory ducts of virgin males. Sperm were pumped into the accessory glands and were later ejaculated with accessory gland secretions during mating. No active mixing of sperm with the accessory gland substance was seen in the lumen of the accessory glands of virgin males. The sperm reservoir became shorter and narrower in mated males and 4 to 5-mated males had the smallest sperm reservoirs.

The accessory glands of male Lu. anthophora were differentiated into an anterior portion filled with light yellow accessory granules, and a posterior portion composed of a band of small sized dark yellow granules. Such differentiation of accessory glands was previously reported for mosquitoes by histological and histochemical studies (Dapples et al. 1974; Adlakha et al. 1976b; Mahmood & Reisen 1982; Mahmood et al. 1986).

In Ae. aegypti, accessory gland substance is ejaculated shortly before the release of sperm (Jones & Wheeler 1965). The MAGS or 'matrone' induces refractoriness to further insemination in mated female of Ae. aegypti (Craig 1967). The mated females, or females injected with matrone are unable to erect their cerci and as a consequence males are unable to grasp them while attempting to mate (Gwadz et al. 1971). The matrone is a protein composed of two subunits α and ß (Fuchs et al. 1968, 1969).

The α fraction alone enhances oviposition but together with ß fraction it prevents insemination (Hiss & Fuchs 1972). Inseminated females, or those injected with matrone digest blood more rapidly than virgin females or those injected with saline (Downe 1975). Accessory gland substance is also necessary for fertilization of eggs. Eggs laid by female mosquitoes earlier mated with males lacking accessory glands did not hatch; although such females had ample sperm in their spermathecae (Adlakha & Pillai 1975, 1976a). Later, Ramalingam & Craig (1976) demonstrated that secretions from the anterior portion of the accessory glands were responsible for rendering the females refractory to further insemination and that the posterior mucin secretions cohered the anterior secretory granules.

The accessory gland substance also induces increased oviposition in _Ae_. _aegypti_ females. No studies are available about the nature of anterior and posterior secretions of the accessory glands of _Lu_. _anthophora_ and further studies are needed.

Male _Lu_. _anthophora_ were capable of inseminating as many as five females within a period of 3 hrs. One 5-day-old virgin male successfully inseminated three females within 2 hrs and spent 52.5 minutes in copula. The reproductive system of 5-mated male was depleted of MAGS, But sperms were still present in the sperm reservoir. Such males transferred large amount of yellow accessory gland substance compared to sperm to the first female. The second female received about equal amounts of sperm and accessory gland substance. The 3rd to 5th females received more sperm and less MAGS. The spermathecae of last mated female actually contained numerous sperm and a few granules of light yellow MAGS.

The present observations were also supported by the comparison of the reproductive systems of 1 to 5-times mated males. The accessory glands of 1-mated males lost both light and dark yellow MAGS and only small amount of sperm; the testes could not be distinguished from that of virgin males. The sperm reservoir of 2-mated males also had numerous spermatozoa but the accessory glands were pale and the posterior band of dark yellow MAGS was very pale. In 3 to 5-mated males the dark yellow accessory gland substance was invisible and accessory glands became smaller. The physiological implication of such changes are not yet known for sand flies.

Multiple insemination is reported to occur in sand flies under laboratory conditions (Chaniotis 1967) and was also observed in _Lu_. _anthophora_. In certain species of mosquitoes, like _An_. _culicifacies,_ multiple insemination is a result of increased density of adults in the cages; no multiple insemination was observed under uncrowded condition in the large cages (Mahmood & Reisen 1980). Females of _Lu_. _flaviscutellata_ (Mangabeira) exhaust their sperm and MAGS supply after first oviposition and remate after oviposition (Ward 1977). Similar behavior was observed in remated Rock Strain of _Ae_. _aegypti_ in females that progressed through a number of gonotrophic cycles. The incidence of remating increased in females that progressed through multiple gonotrophic cycles. Thus, 48 % of the females that laid 6 and 7-times remated, compared to 6 % in females that oviposited only once (Williams & Berger 1980). Similar behavior was observed in field caught _Ph_. _papatasi_ that remated after oviposition (Yuval & Schlein 1986). Mating behavior of _Lu_. _anthophora_ was not observed after oviposition so it is not known if they also behave like other sand flies.

The present study also showed that the use of different structures of male reproductive system as taxonomic tools for identification of male sand flies is not always reliable. Changes induced due to aging and mating can change the ratios of the measurements of different unsclerotized organs.

CHAPTER 4
QUANTITATIVE DETERMINATION OF TRYPSIN- AND CHYMOTRYPSIN-LIKE ENZYMES IN IMMATURE AND ADULT *LUTZOMYIA ANTHOPHORA*

Introduction

The midgut epithelium of immature and adult insects secrete different proteolytic enzymes (Greenberg & Parestsky 1955; Yang & Davies 1968, 1971; Hagenmaier 1970; Akov 1972; Gooding 1972, 1975, 1976; Kunz 1978; Graf & Briegal 1982, 1985; Borovsky 1985, 1986; Sharma et al. 1984; Graf et al. 1986, 1988). Proteolytic enzyme activity varies in different larval instars of the mosquito, *Aedes aegypti* and is very low at larval-pupal ecdysis (Yang & Davies 1971). Whole body extract of *Ae. aegypti* exhibited 12-14 different endopeptidases, with different mobilities on gel electrophoresis (Kunz 1978). Whole body extracts of the house fly maggots, *Musca domestica*, showed lower tryptic activity compared to midgut extracts. This may be due to an inhibitory substance in the body of the maggots (Greenberg & Paretsky 1955).

Information about larval breeding sites of sand flies and about their food in natural habitats is little known (Lewis 1982; Killick-Kendrick 1983). Most of the available information shows that larvae feed on organic detritus and feces of animals (Addis 1945a; Hanson 1961; Perfil'eve 1968; Killick-Kendrick 1978). Detritus has a limited nutritional value due to the small amount of available nitrogen (McNeill & Southwood 1978). Accordingly, high levels of proteolytic enzymes are synthesized in the midguts of detritus feeding larvae of crane flies, caddisflies, and stoneflies (Martin et al. 1980, 1981a, 1981b). High levels of midgut proteolytic enzymes enable an efficient utilization of detritus by the larvae of crane fly _Tipula_ _abdominalis_ (Sharma et al. 1984).

Several proteolytic enzymes are secreted by the midgut epithelium of adult hematophagous insects, before during and after ingesting a blood meal. Following blood feeding midgut secretory cells synthesize active and inactive proteases which are secreted into the lumen of the midgut (Gooding 1975; Graf et al. 1986). To date, no protease precursor has been detected in the midgut of _Ae_. _aegypti_ (Fisk 1950; Shambbaugh 1954), although a claim was recently made that an inactive trypsinogen was synthesized by the secretory cells of the midgut epithelium in _Ae_. _aegypti_ (Graf et al. 1986).

Alkaline proteases are detected 4 hours after ingestion of a blood meal and reach peak activity at 24 to 35 hours depending on the mosquito species (Gooding 1966a, 1966b; Briegal & Lea 1975; Borovsky 1986; Borovsky & Schlein 1988; Graf et al. 1986), but a decline in protease activity occurred in Ae. aegypti 24 hrs after ingestion of a blood meal (Fisk & Shambaugh 1952). The level of proteolytic activity was similar to sugar fed females after complete digestion of the blood meal (Graf & Breigal 1982).

Few reports are available about proteolytic enzymes in adult sand flies (Schlein et al. 1983; Schlein & Romano 1986; Borovsky & Schlein 1987). No information is available about trypsin- and chymotrypsin-like enzymes of their larvae. Lack of information is primarily a result of difficulties in colonization of sand flies and their small size.

Recent advances in the colonization of sand flies (Young et al. 1980) and sensitive methodology for quantitative determination of trypsin- and chymotrypsin-like enzymes in small insects (Borovsky & Schlein 1988) has made it possible to study these enzymes in phlebotomine sand flies. The present study follows the biosynthesis of trypsin- and chymotrypsin-like enzymes in larvae, pupae, newly-emerged, unfed, sugar fed and blood fed female Lu. anthophora.

Materials and Methods

Strain. Lutzomyia anthophora (Addis) was reared in a bioroom under similar condition as described earlier. The temperature was 26 ± 2 C°, relative humidity was 75 ± 5% and the photoperiod was 16:8 (L:D) (chapter 3).

Duration of Larval Instars

To determine the duration of different larval instars, 20 groups of first instar larvae (50 per group) were reared in separate rearing tubes. Ample food was added to each tube on alternating days to avoid competition. Ten larvae were randomly selected and total body length and head capsule width were measured daily at 10X magnification under a dissecting microscope. Changes such as reduction in length of larval body just before molting and condition of larval midguts were also noted.

Quantitative Determination of Trypsin- and Chymotrypsin-like Enzymes

Trypsin- and Chymotrypsin-like enzymes of larvae, pupae and adult females of *Lu*. *anthophora* and larval food were determined according to the methods of Borovsky and Schlein (1988).

Preparation of calibration curves

Porcine pancreatic trypsin type IX (Sigma, St. Louis, MD), was purified as described by Borovsky and Schlein (1988). The enzyme, 95% pure, was free of chymotrypsin activity. A solution of 1 mg/ml was prepared and stored at 4°C. Similarly, bovine pancreas type II chymotrypsin (Sigma) was further purified by utilizing cation exchange chromatography (Walsh & Wilcox 1970). It was crystallized 3 times. The enzyme was free of trypsin contamination and was at least 90-95% pure. A stock solution was prepared and kept at -20°C to ensure stability.

Preparation of larval food homogenate

To find out whether larval food has trypsin- and chymotrypsin-like activity, 3 samples, weighing 10 mg each, were dissolved in 100 µl of 50 mM Tris-HCl buffer containing 0.1 M $CaCl_2$, pH 7.9, and compared to controls containing 100 µl of Tris-HCl buffer.

Preparation of larvae and pupae midguts

One day following egg hatching, 20 midguts of randomly isolated larvae were dissected in normal saline, then immediately transferred to 50 mM Tris-HCl buffer, pH 7.9 containing 0.1 M $CaCl_2$. Midguts were then stored at -20°C. Later, midguts were thawed to room temperature and homogenized with a glass homogenizer. The homogenates were centrifuged at 4°C at 14,000g for 20 minutes. Supernates were divided into two 50 µl portions, each transferred into a separate microcentrifuge tube and stored at -20°C. The above procedure was repeated daily from egg hatching to pupation. Since it was not possible to dissect the midguts of pupae without the loss of midgut contents, 20 pupae were homogenized at 1- and 8-days after pupation. Three samples of larval midguts were collected daily for 17 days. Similarly, three samples of 1- and 8-day-old pupae were also collected.

Preparation of midgut homogenates of adult females

Three groups of midguts (20 per group) of newly emerged, 1-, 2-, and 3-day-old sugar fed females were dissected in saline and stored at -20°C. Sugar fed (3-day-old) females were offered a blood meal on an anesthetized hamster. Midguts of blood fed females were dissected at the following intervals 0, 2, 4, 12, 18, 24, 30, 38, 48, 72, and 96 hrs after blood feeding. The midguts were stored and processed as above.

Determination of [1,3-^{3}H]DIP-trypsin and -chymotrypsin-like derivatives

[1,3-^{3}H]DIP-trypsin- and chymotrypsin-like derivatives were prepared by incubating dissolved larval food, and homogenates of midguts of larvae, pupae and females *Lu. anthophora* with 1 µl of [1,3-^{3}H]DFP (5 µCi in 1 µl, specific activity 35 µCi /mM, Amersham) for 24 hours at 4°C. Similarly, different dilutions (0-5 µg) of porcine pancrease trypsin and bovine pancreas chymotrypsin and controls containing 100 µl of Tris-HCl buffer, 0.1 M $CaCl_2$, pH 7.9, were also incubated with 1 µl of [1,3-^{3}H]DFP as above.

Phosphorylation of serine residue at the active site resulted in irreversible conversion of [1,3-^{3}H]DFP into [1,3-^{3}H]DIP-trypsin- or chymotrypsin-like derivatives (Jansen et al. 1949; Dixon et al. 1958; Borovsky & Schlein 1988). To assay for trypsin activity, chymotrypsin activity was inhibited by the addition of 5 µl of 10 mM tosylamide-2-

phenyl chloromethyl ketone (TPCK) per 50 µl of midgut homogenate. TPCK (10mM) inhibited 95% of [1,3-^{3}H]DIP-chymotrypsin formation (Borovsky & Schlein 1988). Thus, only [1,3-^{3}H]DIP-trypsin-like derivatives were assayed.

Aliquots (5-50 µl) of the different dilutions (0-5 µg) of porcine pancreas trypsin, bovine pancreas chymotrypsin (0-5 µg), midgut homogenates of larvae, pupae and female adults, dissolved food (controls) and buffer controls were adsorbed onto squares of filter paper (4 X 4 cm). The filter papers were washed for 15 minutes in 200 ml of 10% cold TCA, replaced twice with 5% TCA for 15 minutes and followed by a 5 minute wash in absolute alcohol. The papers were dried at 50°C and added into tubes containing 3 ml of liquid scintillation (Liquifluor) and radioactivity was counted in a liquid scintillation counter (Tracor Analytical Model 6892).

Polyacrylamide Gel Electrophoresis (PAGE) and Fluorography

Polyacrylamide gel electrophoresis of [1,3-^{3}H]DIP-trypsin-like derivatives of midgut homogenates were run in a modified Leammli system (Leammli 1970) as described by Borovsky (1986) and Borovsky and Schlein (1987, 1988). A slab gel (1.5 mm thick, 15 cm long), containing a staking gel of 3% (W/V) (3.0 cm) polyacrylamide and 0.125 M TRIS-HCl, pH 6.8, and a separating gel of 10% (W/V) (12 cm) polyacrylamide and 0.375 M TRIS-HCl, pH 8.8 was used during electrophoresis.

To follow the synthesis of trypsin- and chymotrypsin-like enzymes in larvae, pupae and adult Lu. anthophora, 7 gels were casted. The first four gels contained 17 midguts of larvae and pupae. In each gel several lanes were run simultaneously containing either trypsin- and chymotrypsin-like enzymes or only trypsin-like enzymes.

The fifth gel contained trypsin- and chymotrypsin-like isozymes of midgut homogenates (9 midgut equivalents per lane) of larvae (2nd-4th instar), 1-day-old pupae, newly emerged adults, 1-day-old sugar fed, 2-day-old sugar fed and 3-day-old sugar fed Lu. anthophora females.

The 6th gel was run to determine both trypsin- and chymotrypsin-like isozymes of blood fed females at 0, 2, 4, 12, 18, 24, 30, 38, 48, 72, and 96 hrs after blood feeding. Each lane contained 5 midgut equivalents.

The seventh gel was run to differentiate between trypsin- and chymotrypsin-like derivative in blood fed females. Midgut homogenates (9 midgut equivalents) 24, 48, 72, and 96 hrs after blood feeding were studied. Midgut homogenate containing trypsin- and chymotrypsin-like isozymes and only trypsin-like isozymes were run next to each other.

Poly acrylamide gel electrophosis was run at 25 volts for 1/2 hour and at 35 volts for 1 hour. Gels were stained in 0.1% (W/V) comassie brilliant blue R-250 for 15 minutes and were destained in a mixture of acetic acid, methanol and water respectively (10% glacial acetic acid : 25% methanol) for 1 hr. Washed in glacial acetic acid methanol (10% : 10%) solution for one hour and destained over night in glacial acetic acid : methanol (5% : 7%) solution.

Gels were then incubated with Dimethyl sulfoxide (DMSO)(80ml) for one hour. The solution was replaced with 22% 2,5-diphenyl oxazole (PPO) in dimethyl sulfoxide (DMSO) and incubated for 1 hour. Following incubation gels were rinsed with distilled water and were kept in water for 30 minutes to facilitate complete precipitation of PPO. The gels were dried at 60°C for 3 hrs under vacuum on a filter paper in a gel drier (Model 583; BioRad, Richmond Calif. USA). Dry gels were exposed for 4-20 days to X-ray films at -70°C and were developed in a dark room.

Statistical Analysis

A one way analysis of variance was performed to determine significant changes in the length of larval body and width of their head capsule at different ages (Sokal & Rholf 1981). Larval instars were differentiated after applying Duncan's new multiple range test (Sokal & Rholf 1981). A quadratic (or second-order polynomial) model was used to describe the changes in length of the body and width

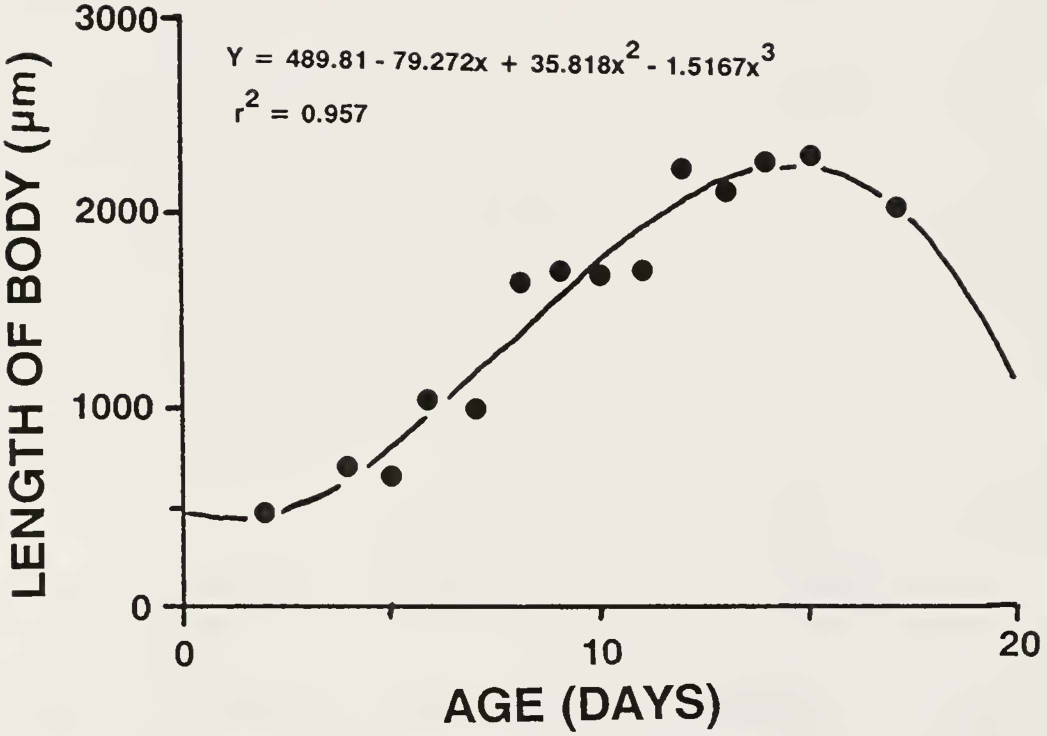

Figure 4-1. Relationship between the body length and age (days) in the larvae of Lutzomyia anthophora.

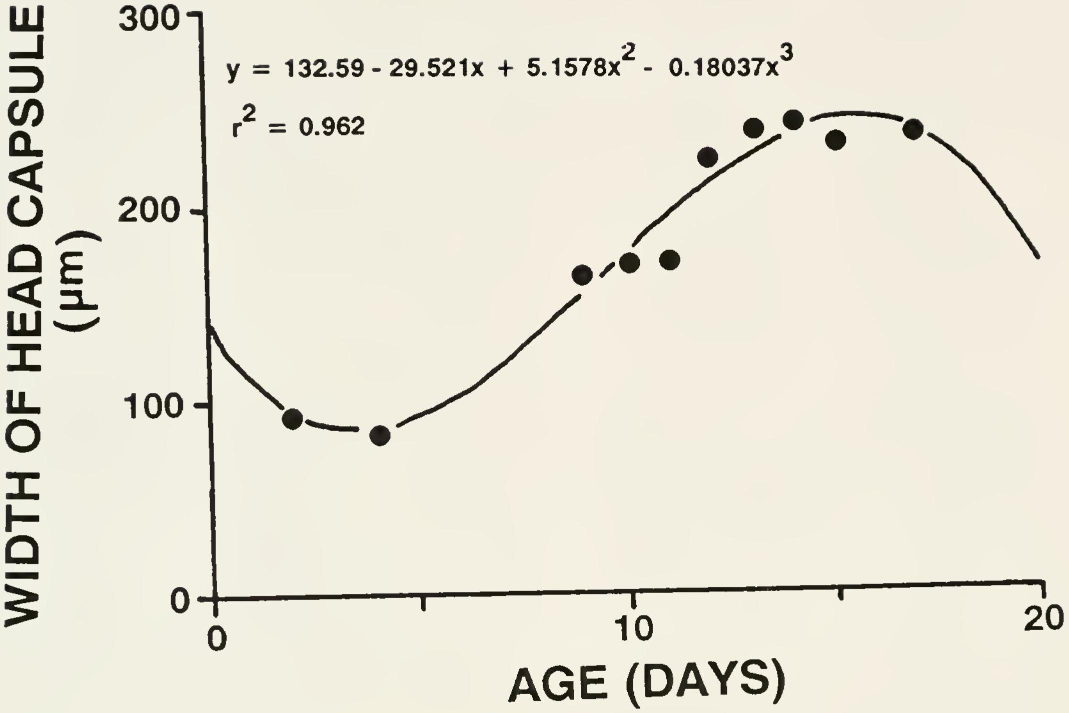

Figure 4-2. Relationship between head capsule width and age (days) of the larvae of Lutzomyia anthophora.

of the head capsule of larvae as a function of increasing age (Mark 1982).

Simple linear regression was performed between the amount of trypsin and chymotrypsin (ng/100 µl) and total cpm of [1,3-^{3}H]DIP-trypsin- and -chymotrypsin-like derivatives. Trypsin- and chymotrypsin-like enzymes of various midgut homogenates were quantitatively determined by using the linear portion of the calibration curve.

Significant changes in the activity of serine proteases of immature or female _Lu_. _anthophora_ were determined by using Duncan's new multiple range test. Significant changes in the quantities of trypsin- and chymotrypsin-like enzymes synthesized by the midguts of different larval instars, pupae and females were determined similarly (Sokal & Rholf 1981). Percentage of trypsin activity was determined for both immature and adult stages.

Results

Duration of Different Larval Instars

A significant correlation was found between larval body length and age (r^2 = .96) (Fig. 4-1), and width of the head capsule and age of _Lu_. _anthophora_ (r^2 = .96) (Fig. 4-2). Width of the head capsule increased significantly as the larvae grew older (α = 0.05, F = 115.29 and P < 0.0001). Smallest width of head capsule was found in 2 to 5-day-old

Table 4-1. Age related changes in the width of head capsule and the length of body of different larval instars of Lu. anthophora.

Larval Instar	Age (Days)	Head capsule (Width)	Body (Length)
		$\overline{\mu m}$ ± SE$\bar{X}$*	$\overline{\mu m}$ ± SE$\bar{X}$*
First	2	90.0 ± 4.1^{E}	470.0 ± 57.8^{E}
	4	84.0 ± 3.8^{E}	657.5 ± 27.3^{D}
	5	100.0 ± 0.0^{E}	705.0 ± 25.8^{D}
Second	7	127.5 ± 6.9^{D}	1045.0 ± 36.1^{C}
	8	115.5 ± 7.5^{D}	990.0 ± 44.6^{C}
Third	9	161.5 ± 3.9^{D}	1630.0 ± 41.0^{B}
	10	168.5 ± 6.3^{C}	1685.0 ± 33.7^{B}
	11	169.0 ± 6.2^{C}	1695.0 ± 40.2^{B}
Fourth	12	222.5 ± 5.8^{B}	2220.0 ± 128.5^{A}
	13	239.0 ± 5.4^{AB}	2105.0 ± 89.3^{A}
	14	239.5 ± 5.1^{A}	2260.0 ± 64.5^{A}
	15	215.0 ± 5.2^{B}	2300.0 ± 70.7^{A}
	17	229.4 ± 6.6^{AB}	2244.0 ± 73.3^{A}

*Mean ± S.E.M followed by the same letters were not significantly different when compared by Duncan's new multiple range test ($\alpha = 0.05$, $P < 0.0001$, $F = 115.29$ and $N = 10$ for each age).

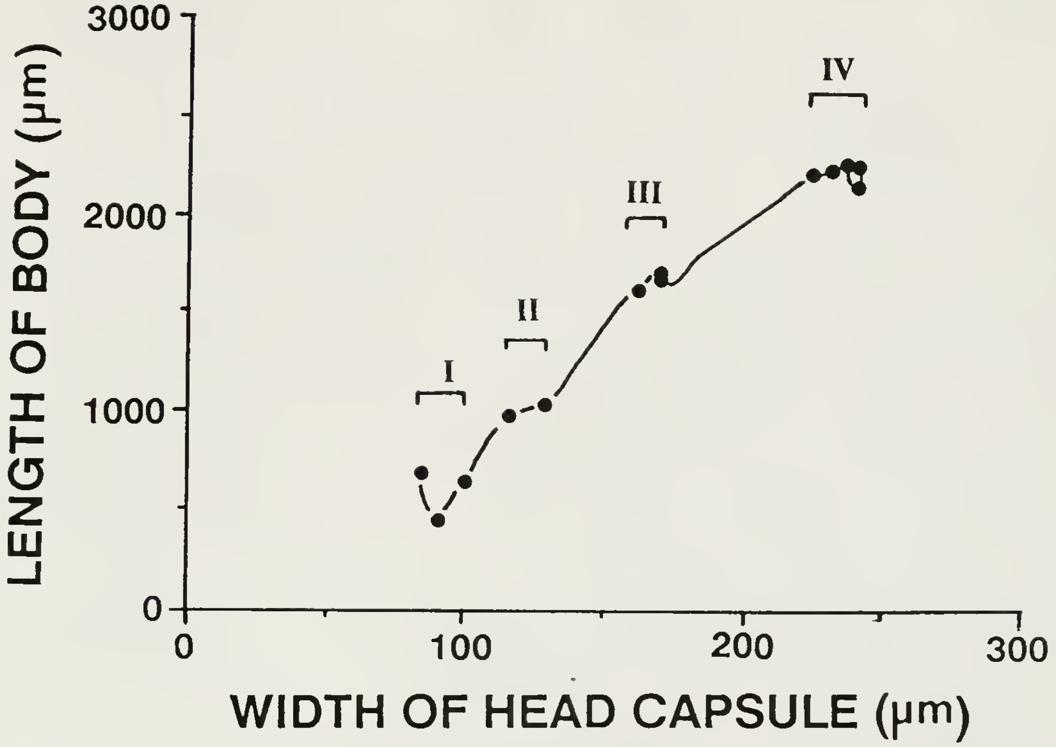

Figure 4-3. Relationship between body length and head capsule width of *Lutzomyia anthophora* larvae.

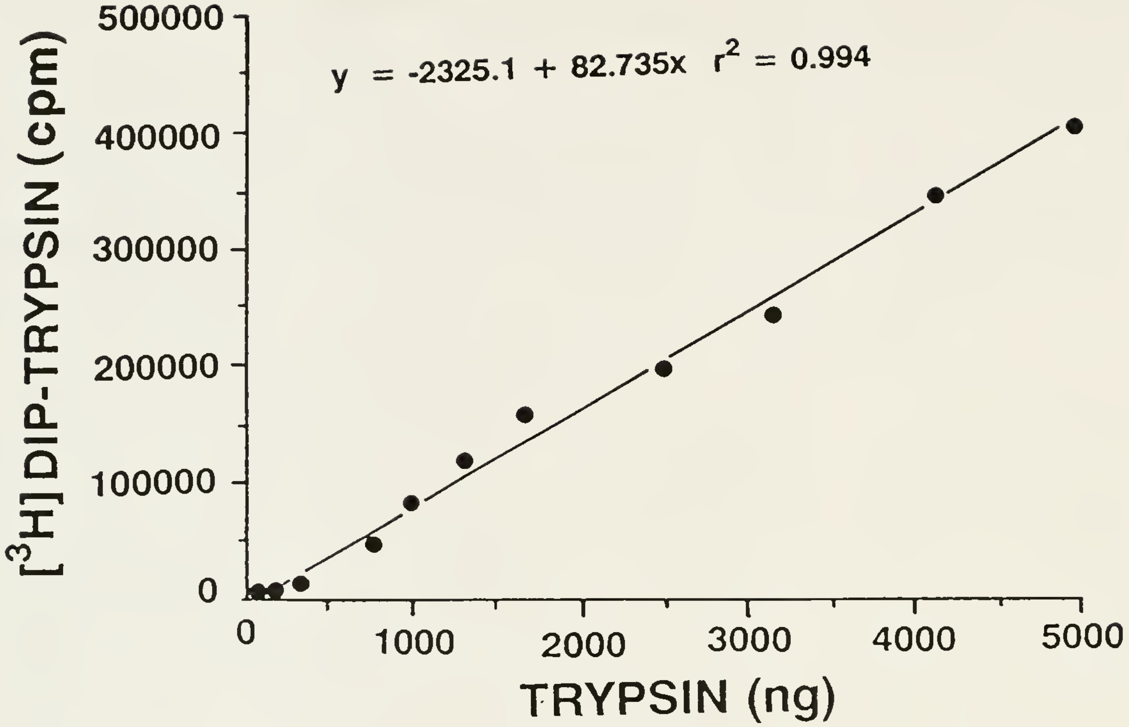

Figure 4-4. Calibration curve of trypsin, showing relationship between trypsin concentration (ng/100µl) and [1,3-^{3}H]DIP-trypsinderivatives.

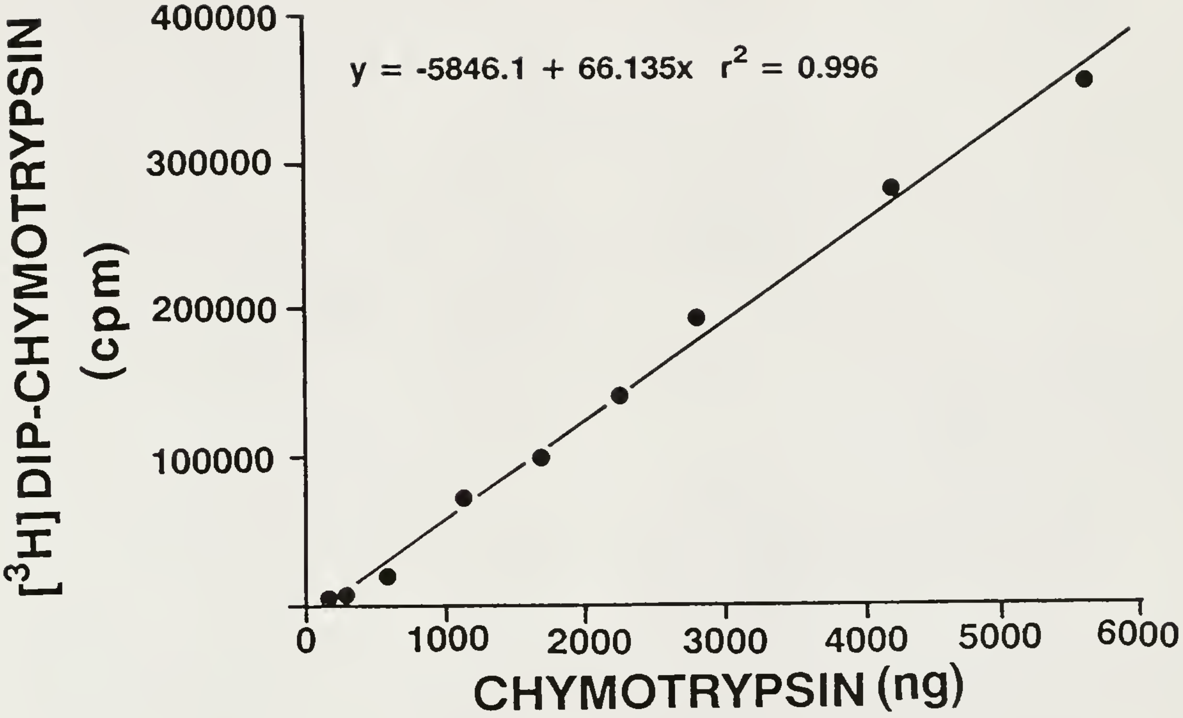

Figure 4-5. Calibration curve for the determination of chymotrypsin, showing relatinoship between chymotrypsin concentration (ng/100 µl) and [1,3-^{3}H]DIP-chymotrypsin derivatives.

first instar larvae and widest head capsule was found in 12 to 17-day-old 4th instar larvae (Table 4-1). The length of larval body increased significantly due to age ($\alpha = 0.05$, $F = 118.14$ and $P < 0.0001$). Smallest length was found in 2-day-old 1st instar larvae, whereas 12 to 17-day-old 4th instar larvae were largest (Table 4-1). Four distinct larval instars were present in Lu. anthophora (Fig. 4-3)(Table 4-1). A slight decrease was observed in the length of larvae just before molting, however these larvae emptied their midguts at this time.

The larvae pupated 15 to 17-days after eclosion from the eggs. Adult ecdysis occurred 8-days after pupation (Table 4-1).

Trypsin- and Chymotrypsin-like Enzymes of Lu. anthophora

Trypsin- and chymotrypsin-like enzymes changed significantly in immature and adult Lu. anthophora as is evident from the following results.

Calibration curves for [1,3-^{3}H]DIP-trypsin and chymotrypsin

A linear relationship was present between the quantities of trypsin (ng) and chymotrypsin (ng) and total cpm of [1,3^{3}H]DIP-trypsin and -chymotrypsin (r^2 = .994 and .996 respectively) (Fig. 4-4 & 4-5). The straight lines thus obtained were used to quantitatively determine the amounts of trypsin- and chymotrypsin-like enzymes of immature and adult Lu. anthophora and the larval food.

Table 4-2. Time course of the production of serine proteases in immature and adult _Lu_. _anthophora_.

Age(Days)		$[1,3-{}^{3}H]$DIP / Midgut $\overline{\text{cpm}}$ ± SE$\bar{X}$* (N)
		LARVAE
1	1st instar	48 ± 20 (3)
2		154 ± 23 (3)
3		279 ± 22 (3)
4		229 ± 34 (3)
5		578 ± 102 (3)
7	2nd instar	1619 ± 149 (3)
8		1974 ± 387 (3)
9	3rd instar	4072 ± 641 (3)
10		3595 ± 155 (3)
11		3603 ± 412 (3)
12	4th instar	5115 ± 684 (3)
13		9202 ± 2426 (3)
14		11745 ± 678 (3)
15		13946 ± 439 (3)
16		14105 ± 1711 (3)
17		1064 ± 242 (3)
		PUPAE
1		885 ± 56 (3)
8		898 ± 43 (3)
		ADULTS (SUGAR FED)
Newly emerged		613 ± 52 (3)
1		630 ± 73 (3)
2		621 ± 107 (3)
3		379 ± 30 (3)

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*Means ± standard error of the mean (sample size).

Table 4-3. Time course of the production of serine proteases in blood fed Lu. anthophora.

Time (Hrs after feeding)	$[1,3\text{-}^3H]$DIP / Midgut $\overline{cpm}$ ± SE$\bar{X}$[*] (N)
	3-DAYS-OLD BLOOD FED
0-hrs	1420 ± 198 (4)
2-hrs	1639 ± 127 (4)
4-hrs	1881 ± 308 (3)
12-hrs	2254 ± 68 (4)
18-hrs	2623 ± 220 (4)
24-hrs	2191 ± 222 (6)
30-hrs	4754 ± 745 (4)
38-hrs	6958 ± 1284 (4)
48-hrs	9054 ± 1217 (6)
72-hrs	20934 ± 5074 (5)
96-hrs	569 ± 370 (3)

[*] Means ± standard error of the mean (sample size).

Enzymatic activity of food

No trypsin- and chymotrypsin-like enzymatic activities were present in larval food (28 cpm / 1µl of larval food homogenate).

Comparison of total serine proteases produced during the life of Lu. anthophora

The activity of serine proteases significantly changed in different larval instars and adults of Lu. anthophora ($\alpha = 0.05$, $F = 21.02$ and $P < 0.0001$). The smallest amount of serine proteases were secreted by 1-day-old larvae (Table 4-2). The maximum serine proteases (Mean cpm/midgut ± SEM) (20,934 ± 574 cpm) were produced by females 72 hrs after blood meal (Table 4-3). During immature development minimum serine proteases were secreted by first instar larvae (259 ± 48 cpm). The amount of serine proteases increased in 2 to 16-day-old larvae and a decline in the amount of serine proteases occurred in 17-day-old larvae just before pupation (Table 4-2).

The amounts of serine proteases produced by first and second instar larvae (1,796 ± 201 cpm), pupae (569 ± 370 cpm), 0 to 2-day-old females (621 ± 40 cpm), 3-day-old sugar fed females (379 ± 30 cpm), and females 96 hrs after blood meal were not significantly different. Third instar larvae produced 3,756 ± 238 cpm of serine proteases and it was not significantly different from the amount produced by females

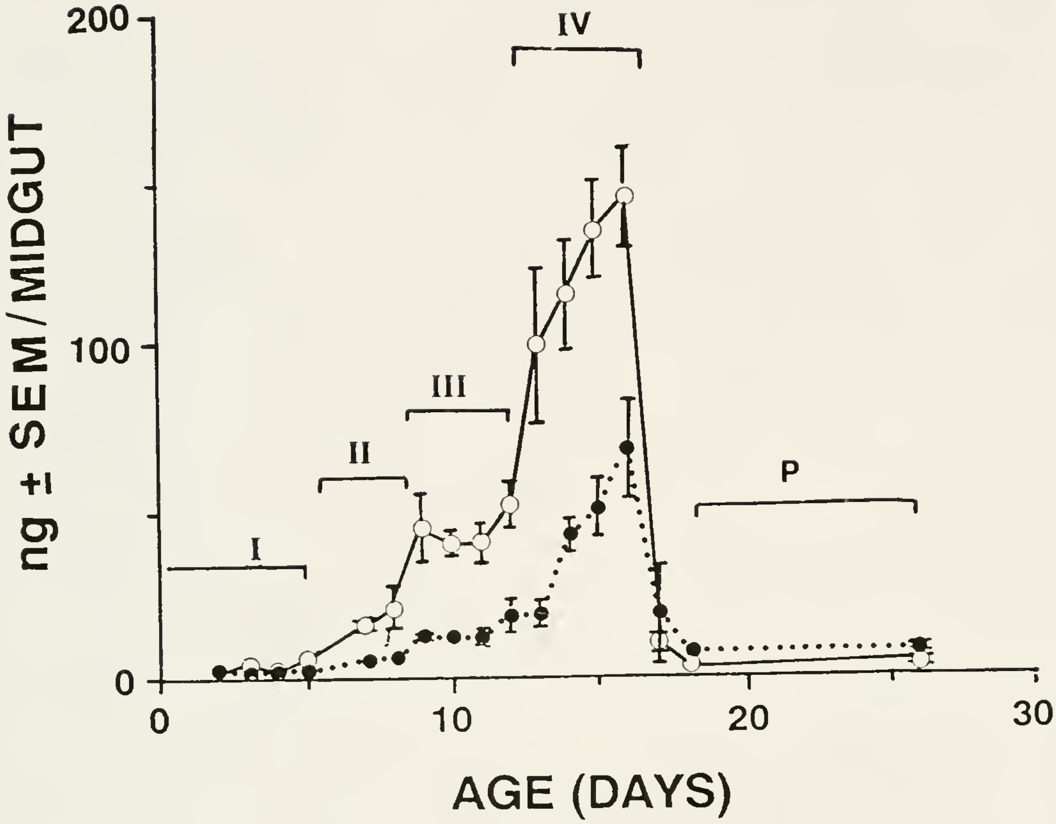

Figure 4-6. Time course of the changes in trypsin- and chymotrypsin-like enzyme concentrations (ng/midgut) of larvae and pupae of Lu. anthophora. Standard error of mean are shown by bars unless these are too small to be shown, (n = 3 for each age). P = Pupae, I, II, III, and IV denote different larval instars. ○——○ chymotrypsin and ●.....● trypsin.

12-24 hrs after the blood meal (2,332 ± 120 cpm) and 30-38 hrs after the blood meal (3,756 ± 238 cpm).

Similarly, no significant difference was found in the amount of total serine proteases produced by fourth instar larvae (9,196 ± 1,236 cpm) and females 48 hrs after blood meal (9,054 ± 1,217 cpm) (Tables 4-2, 4-3).

Quantitative changes in trypsin- and chymotrypsin-like enzymes in *Lu. anthophora*

Significant difference was found between trypsin equivalents (Mean ± SEM ng) secreted in different larval instars and pupae of *Lu*. *anthophora* ($\alpha = 0.05$, $F = 12.97$, $P < 0.0001$) (Fig. 4-6). A continuous increase in the amount of trypsin-like enzymes was observed in 2 to 16-day-old larvae (Fig. 4-6). Trypsin concentration (ng) secreted by first instar (1.13 ± 0.05 ng), second instar (5.57 ± 0.57 ng), third instar (11.84 ± 0.46 ng) larvae, and pupae (7.54 ± 0.95 ng) were not significantly different. Fourth instar larvae had maximum trypsin (36.48 ± 5.74 ng). Smallest quantity of trypsin was secreted by the midgut of 2-day-old first instar larvae and maximum quantity was secreted by 16-day-old larvae (Fig. 4-6). Trypsin concentrations secreted by 17-day-old larvae decreased suddenly due to absence of food in their midguts. Since no feeding occurred during the pupal stage, no increase in the quantities of trypsin was observed (Fig. 4-6).

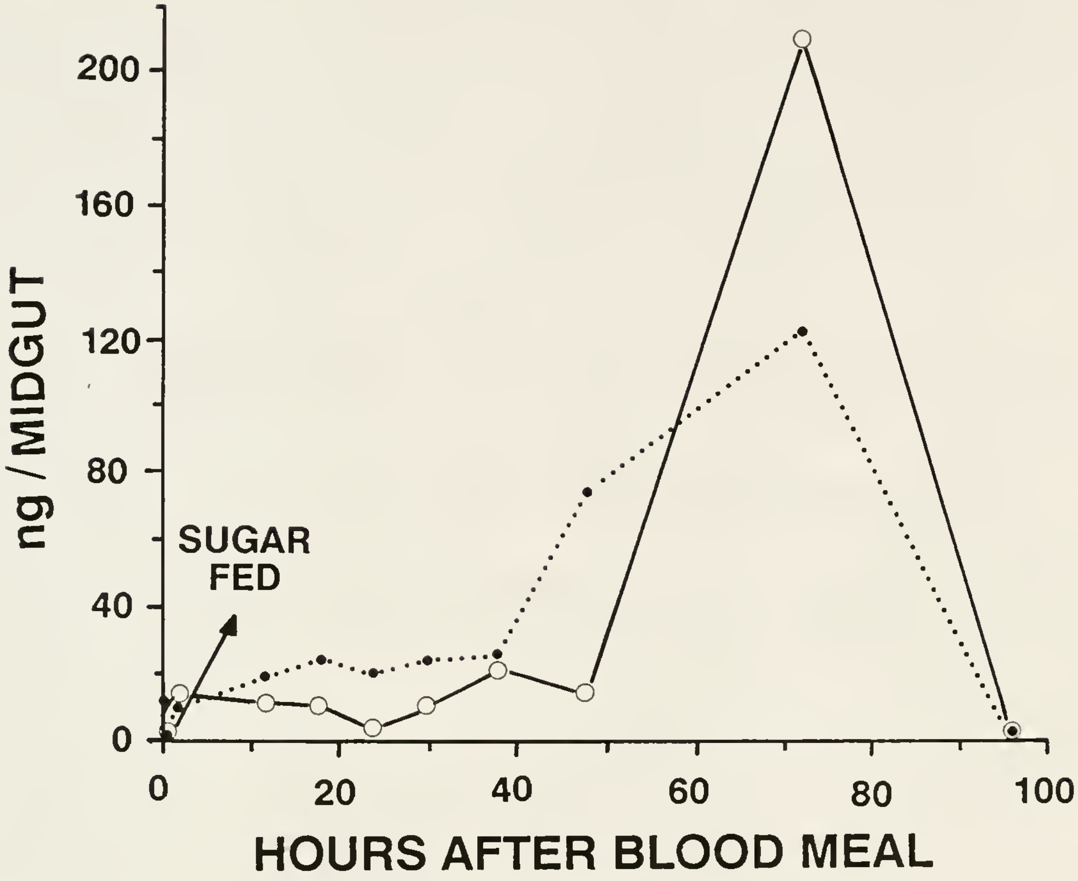

Figure 4-7. Time course of the changes in trypsin- and chymotrypsin-like midgut equivalents (ng/midgut) of 3-days old sugar fed and 3-days old blood fed females of Lu. anthophora at different hours after blood feeding. ●.....● trypsin and ○——○chymotrypsin.

Significant difference was present in the quantities of chymotrypsin (Mean ± SEM ng) secreted by the midguts at different stages of immature Lu. anthophora ($\alpha = 0.05$, $F = 17.83$, and $P < 0.0001$). Continuous increase was observed in quantities of chymotrypsin in 2 to 16-day-old larvae. A sudden decrease in secretion of chymotrypsin occurred in 17-day-old fourth instar larvae (Fig. 4-6).

No significant difference was present in the quantities of chymotrypsin secreted by 2 to 5-day-old first instar larvae (3.21 ± 0.53 ng), 1 to 8-day-old pupae (3.38 ± .51 ng) and 7 to 8-day-old second instar larvae (18.73 ± 2.89 ng). The amount of chymotrypsin secreted by third instar larvae (41.97 ± 3.50 ng) was significantly more than the first instar larvae and pupae. Two fold more chymotrypsin was secreted by fourth instar larvae (92.68 ± 12.54 ng). Maximum amount of chymotrypsin was secreted by the midguts of 16-day-old larvae (144.19 ng) and the smallest amount was secreted by 2-day-old larvae (1.41 ng)(Fig. 4-6).

The amount of trypsin- and chymotrypsin-like enzymes increased significantly after blood ingestion (Fig. 4-7). Larger quantities (ng) of chymotrypsin compared to trypsin were secreted by the midguts of 2-day-old sugar fed (5.06 ng), 3-day-old sugar fed (3.35 ng) and females that blood fed 72 hrs earlier (209.19 ng)(Fig. 4-7).

The amount of trypsin increased shortly after blood feeding and reached a maximum amount after 72-hrs (121.98 ±

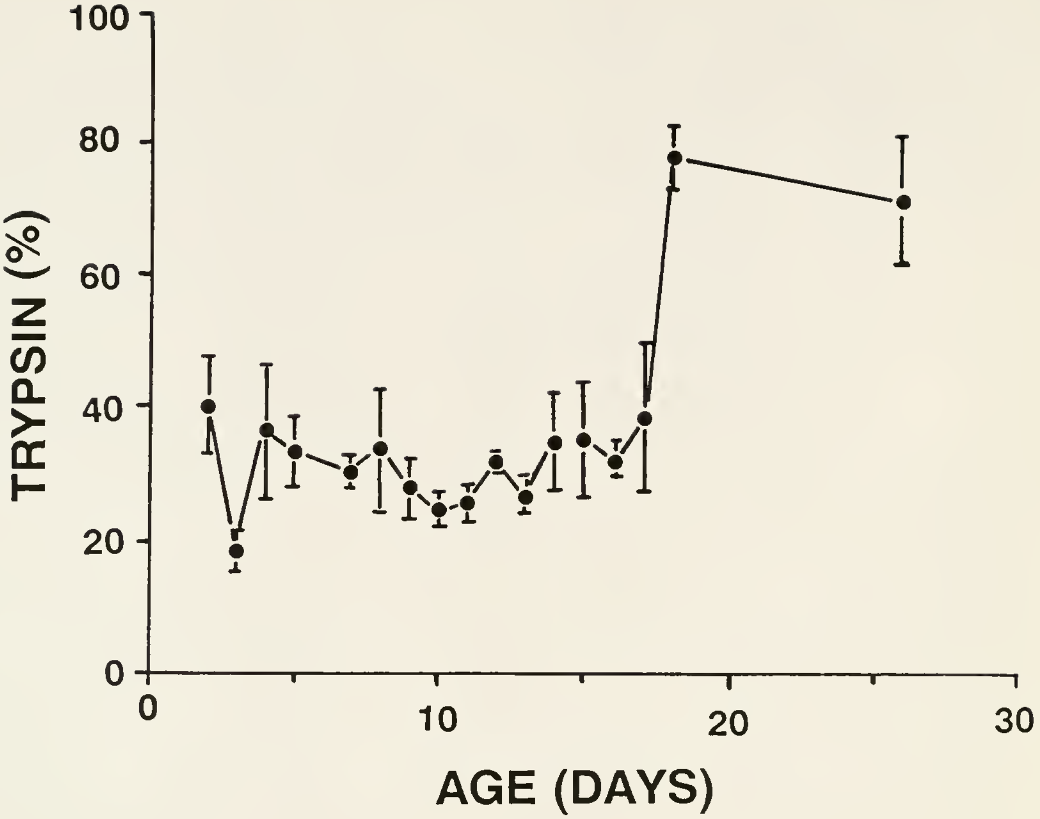

Figure 4-8. Time course of the changes in trypsin ratio compared with other serine proteases (% per midgut) of larvae and pupae of Lu. anthophora. Standard error of mean is represented by bars (N = 3 for each age).

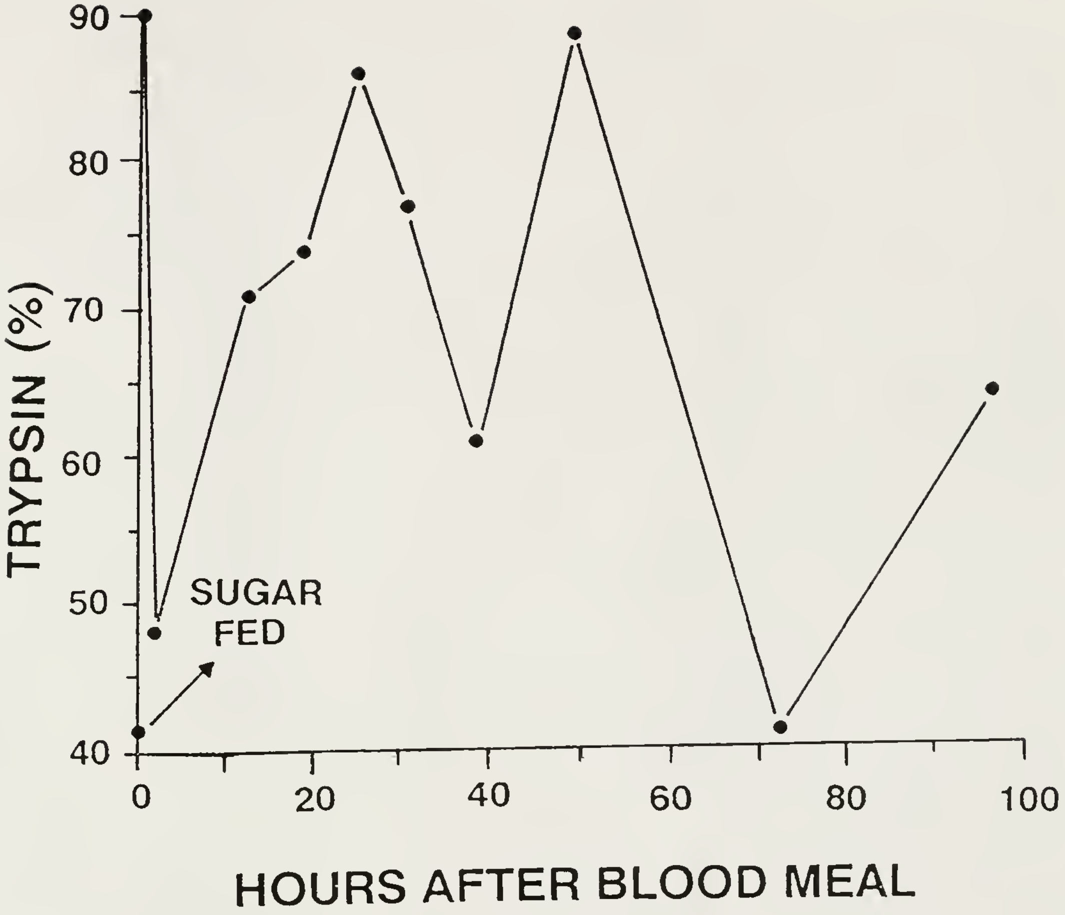

Figure 4-9. Time course of the changes in trypsin ratio compared to total serine proteases (%/midgut) of 3-day-old blood fed *Lutzomyia anthophora* females. Females were dissected at different hours after the ingestion of a blood meal.

10.93 ng)(Fig. 4-7). Although the amount of chymotrypsin also increased after blood feeding, there was only a slight change in the quantity of chymotrypsin in females within first 48 hrs after blood feeding (Fig. 4-7). The amounts of both trypsin and chymotrypsin decreased significantly 96-hrs after blood feeding (Fig. 4-7).

More trypsin and chymotrypsin were secreted by females 72-hrs after blood ingestion than that of 4th instar larvae.

Time course of trypsin activity in the life of *Lu.anthophora*

The ratio of trypsin compared to other serine proteases (%) changed significantly in immature stages of *Lu. anthophora* ($\alpha = 0.05$, $F = 4.80$, $P < 0.0001$) (Fig. 4-8). Lowest trypsin ratio (%) was present in 2-day-old larvae and the highest trypsin ratio (%) was in 1 to 8-day-old pupae. Although, trypsin midgut equivalents (ng) increased significantly in 2 to 16-day-old larvae, the trypsin ratio (%) did not change significantly (Figs. 4-6 & 4-8). A very small quantity of trypsin was present in the pupae but their trypsin ratio (%) was significantly higher (Figs. 4-6 & 4-8).

Very high trypsin ratio (%) was observed just after blood feeding in 3-day-old females, although very small quantity of trypsin was present at this stage (Figs. 4-7 & 4-9). Trypsin ratio (%) declined at 2 hrs but again increased at 12-24 hrs (Fig. 4-9). It declined at 30-38-hrs and peaked again 48 hrs after blood feeding. Minimum

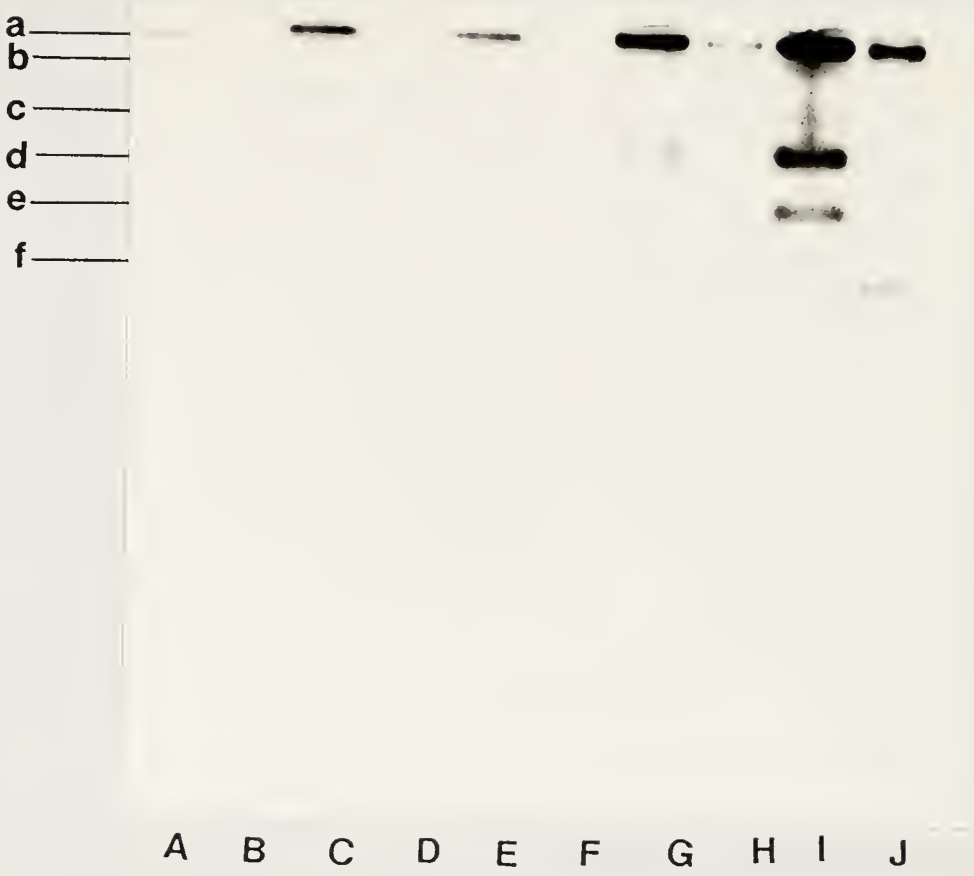

Figure 4-10. PAGE Fluorography of [1,3-^{3}H]DIP-trypsin- and -chymotrypsin-like isozymes of first and second instar larvae of Lu. anthophora. Each lane was run with 17 midgut equivalents. The X-ray film was exposed to the gel for 7 days at -70°C. Lanes A, C, E, G and I contain trypsin- and chymotrypsin-like isozymes. Lanes B, D, F, H and J contain only trypsin-like isozymes. Lanes A-H were run with midgut homogenates of first instar larvae and lanes I-J were run with midgut homogenates of 2nd instar larvae. (A) 2-days (2,618 cpm), (B) 2-days (1,020 cpm), (C) 3-days (4,743 cpm), (D) 3-days (1,496 cpm), (E) 4-days (3,893 cpm), (F) 4-days (3,247cpm), (G) 5-days (9,826 cpm), (H) 5-days (3,247 cpm), (I) 7-days (27,523 cpm) and (J) 7-days (8,449 cpm).

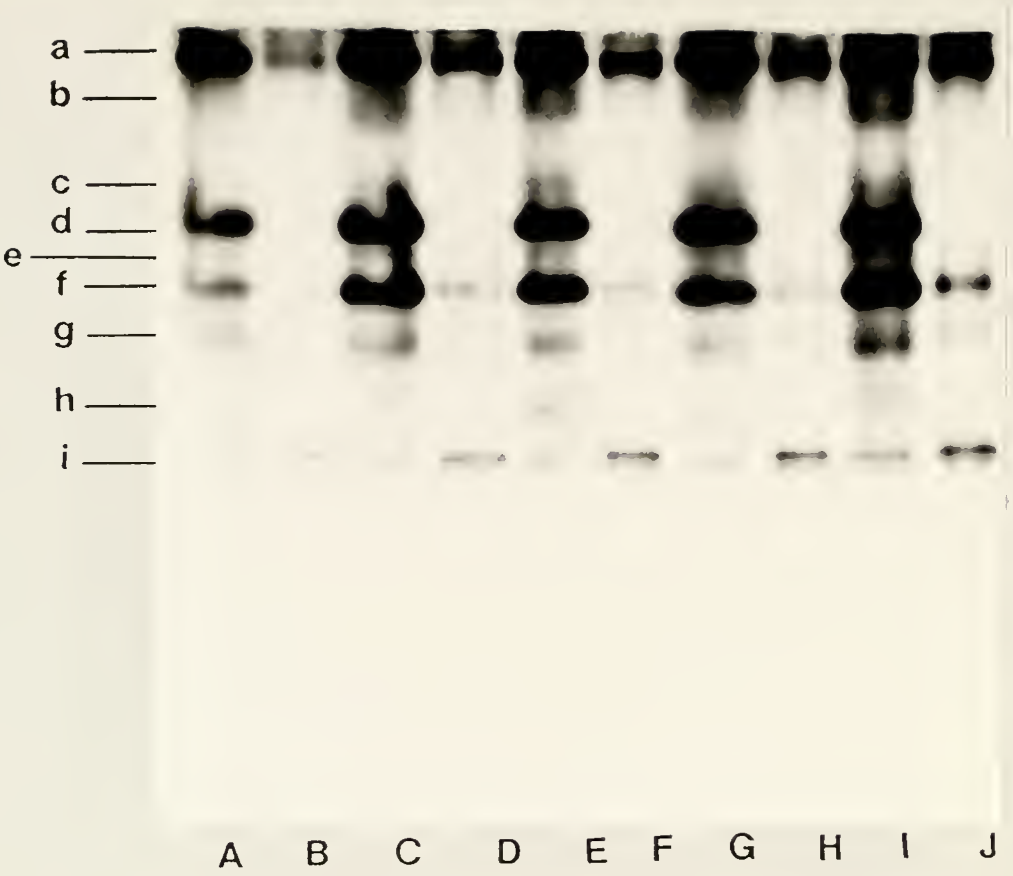

Figure 4-11. PAGE Fluorography of [1,3-^{3}H]DIP-trypsin- and -chymotrypsin-like isozymes of second-fourth instar larvae of Lu. anthophora. Each lane was run with 17 midgut equvialents. The X-ray film was exposed to the gel for 7-days at -70°C. Lanes A, C, E, G and I contained trypsin- and chymotrypsin-like isozymes. Lanes B, D, F, H, and J had only trypsin-like isozymes. Lanes A-B were run with midgut homogenates of second instar larvae, lanes C-H were run with midgut homogenates of 3rd instar larvae and lanes I and J were run with midgut homogenates of 4th instar larvae. (A) 8-day-old (33,558 cpm), (B) 8-day-old (10,166 cpm), (C) 9-day-old (69,224 cpm), (D) 9-day-old (18,292 cpm), (E) 10-day-old (61,115 cpm), (F) 10-day-old (15,045 cpm), (G) 11-day-old (61,251 cpm), (H) 11-day-old (15,555 cpm), (I) 12-day-old (86,955 cpm) and (J) 12-day-old (27,897 cpm).

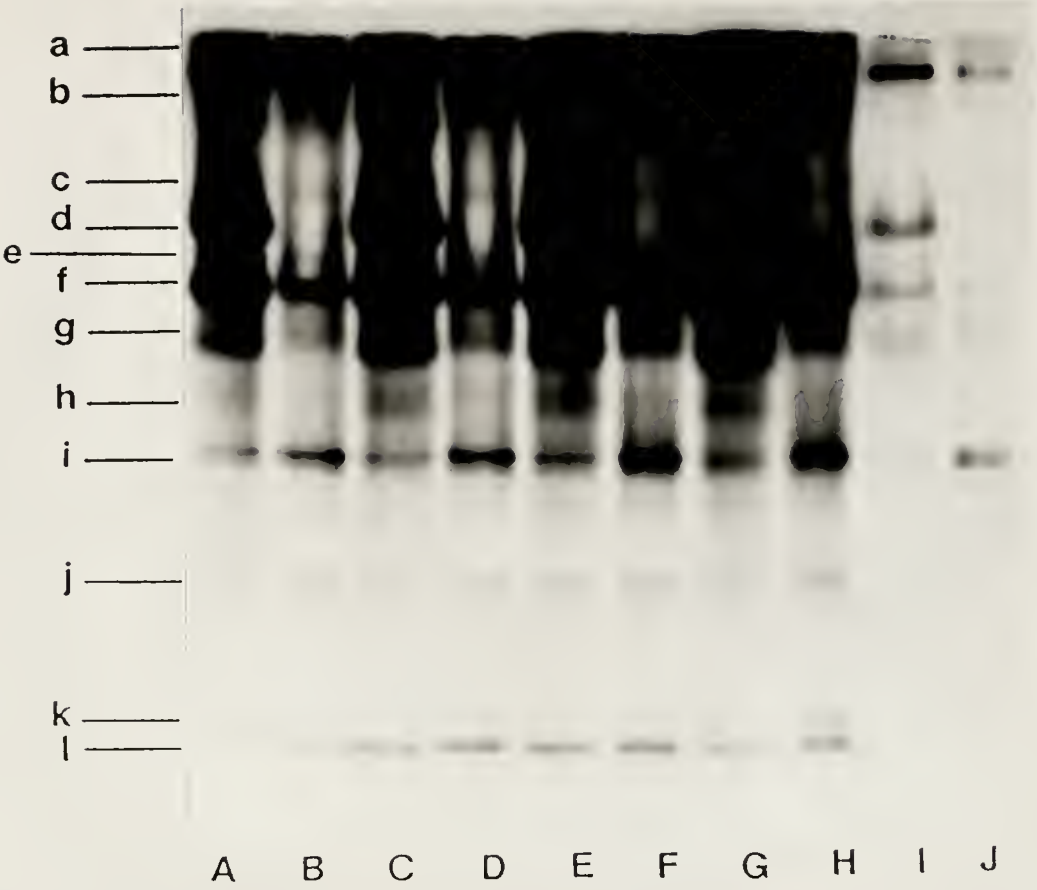

Figure 4-12. PAGE Fluorography of [1,3-^{3}H]DIP-trypsin- and -chymotrypsin-like isozymes of the fourth instar larvae of *Lu. anthophora*. Each lane was run with 17 midgut equivalents. The X-ray film was exposed to the gel for 7 days at -70°C. Lanes A, C, E, G and I contained trypsin- and chymotrypsin-like isozymes. Lanes B, D, F, H and J had only trypsin-like isozymes. Lanes I-J contain fourth instar larvae before molting. (A) 13-day-old (156,434 cpm), (B) 13-day-old (43,401 cpm), (C) 14-day-old (199,665 cpm), (D) 14-day-old (69,411 cpm), (E) 15-day-old (237,082 cpm), (F) 15-day-old (84,541 cpm), (G) 16-day-old (239,782 cpm), (H) 16-day-old (76,364 cpm), (I) 17-day-old (94,976 cpm) and (J) 17-day-old (6,168 cpm).

trypsin ratio (%) was present 72 hrs after blood feeding and was similar to that in 3-day-old sugar fed females (Fig. 4-9). Tryptic ratio was higher in the blood fed females compared to that of the larvae (Figs. 4-7 & 4-8).

Polyacrylamide Gel Electrophoresis and Fluorography of Trypsin- and Chymotrypsin-like Isozymes

The following account presents different isozymes of immature and adult *Lu. anthophora*.

Different isozymes of immature stages

A total of 12 trypsin- and chymotrypsin-like isozymes were detected in different larval instars of *Lu. anthophora* (Figs. 4-10, 4-11, 4-12). The first instar larvae had only two isozymes, represented by major band "a" and minor band "c". Band "a" and "c" were chymotrypsin-like enzymes and were inhibited by TPCK (Fig. 4-10, lanes A-F).

Second instar larvae had seven different trypsin- and chymotrypsin-like isozymes (Fig. 4-10, lanes I & J and Fig. 4-11, lanes A & B). The isozymes separated into 2 major bands (a & d) and 5 minor bands (b, e, f, g, & i). Band "a" either had both trypsin- and chymotrypsin-like activity or was not completely inhibited. It appeared as a thin band after the inhibition of chymotrypsin. Minor bands "f" and "g" were of trypsin-like isozymes (Fig 4-10, lanes I & J and Fig. 4-11, lanes A & B).

Third instar larvae contained 8 different trypsin- and chymotrypsin-like isozymes (Fig. 4-11, lanes C-H). There were 3 major bands (a, d, & f) and 5 minor bands (b, c, e, g, h & i). Major band "d" was chymotrypsin-like isozyme, however, major bands "a" and "f" also contained trypsin-like activity. Minor band "e" was chymotrypsin-like isozyme (Fig. 4-11, lanes D, F, & H). Trypsin-like activity was associated with one major band (a) and 3 minor bands (f, g & h) (Fig. 4-11, lanes D, F & H).

Fourth instar larvae contained high trypsin- and chymotrypsin-like activities (Table 4-2, Fig. 4-12). Peak activity was found in 15 to 16-day-old larvae. A sharp decline was observed in 17-day-old larvae (Table 4-2, Fig. 4-12, lanes E-J). Fourth instar 12 to 16-day-old larvae exhibited 12 different trypsin- and chymotrypsin-like isozymes (Fig. 4-12), with 6 major bands (a, b, d, f, g, & i) and 6 minor bands (c, e, h, j, k & l). Bands "a, b, f, g, i, j, k & l" were trypsin-like (Fig. 4-12, lanes B & D). Long incubation with X-ray film was necessary to highlight all the minor bands in late fourth instar larvae. Thus, it was difficult to distinguish between trypsin- and chymotrypsin-like isozymes in 15 to 16-day-old larvae (Fig. 4-12, lanes E-H). Late fourth instar larvae had very small amount of trypsin- and chymotrypsin-like isozymes (Table 4-2, Figs. 4-6 & 4-12, lanes I & J).

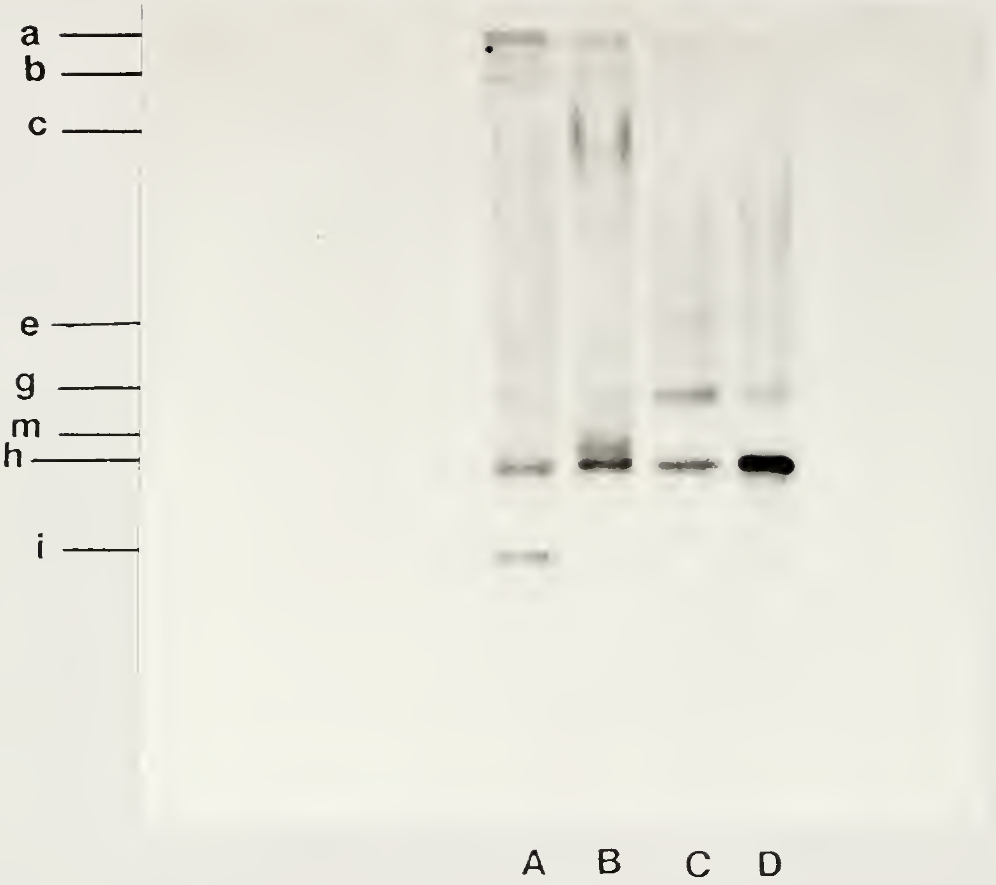

Figure 4-13. PAGE Fluorography of [1,3-^{3}H]DIP-trypsin- and -chymotrypsin-like isozymes of the pupae of Lu. anthophora. Each lane contained 17 midgut equivalents. The X-ray film was exposed to the gel for 7 days at -70°C. Lanes A and C had trypsin- and chymotrypsin-like isozymes. (A) 1-day-old (15,045 cpm), (B) 1-day-old (12,070 cpm), (C) 8-day-old (15,266 cpm) and (D) 8-day-old (10,999 cpm).

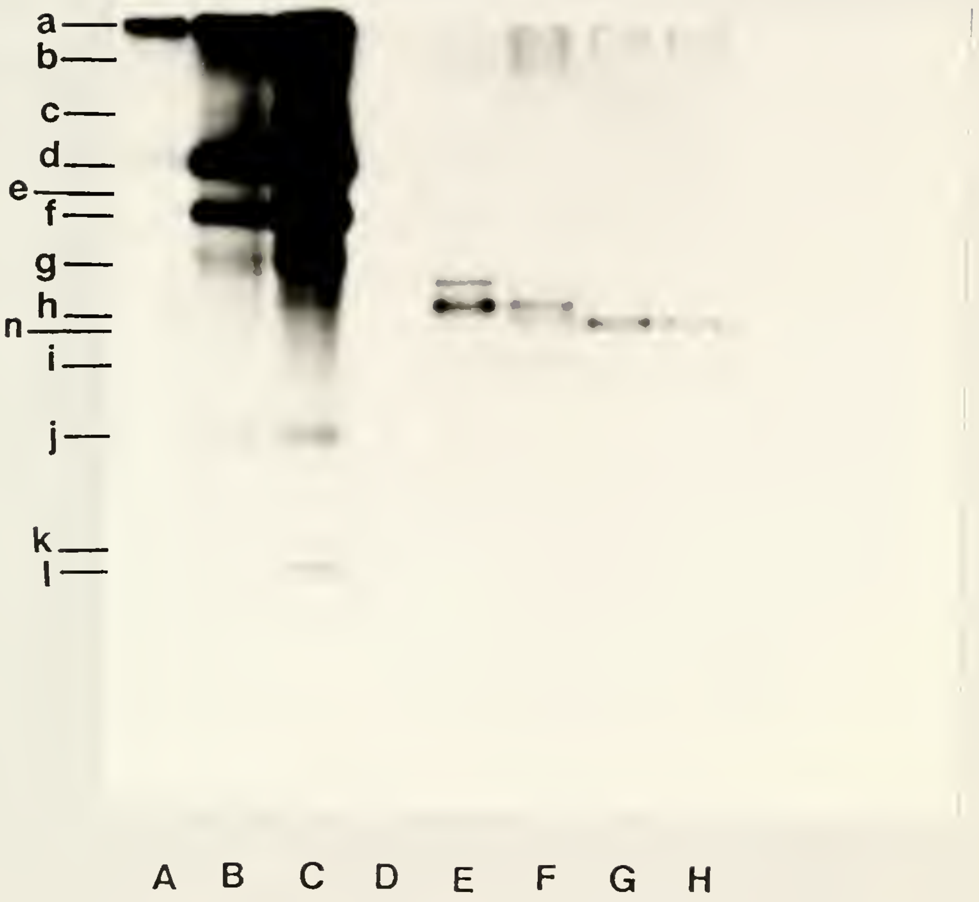

Figure 4-14. PAGE Fluorography of [1,3-^{3}H]DIP-trypsin- and -chymotrypsin-like isozymes of larvae, pupae and females of Lu. anthophora. Each lane contained 9 midgut equivalents. The X-ray film was exposed to the gel for 20 days at -70°C. (A) Second instar larvae (3,840 cpm), (B) Third instar larvae (46,671 cpm), (C) Fourth instar larvae (69,969 cpm), (D) 1-day-old pupae (195 cpm), (E) Newly-emerged females (613 cpm), (F) 1-day-old sugar fed females (630 cpm), (G) 2-day-old sugar fed (621 cpm) and (H) 3-day-old sugar fed (379 cpm).

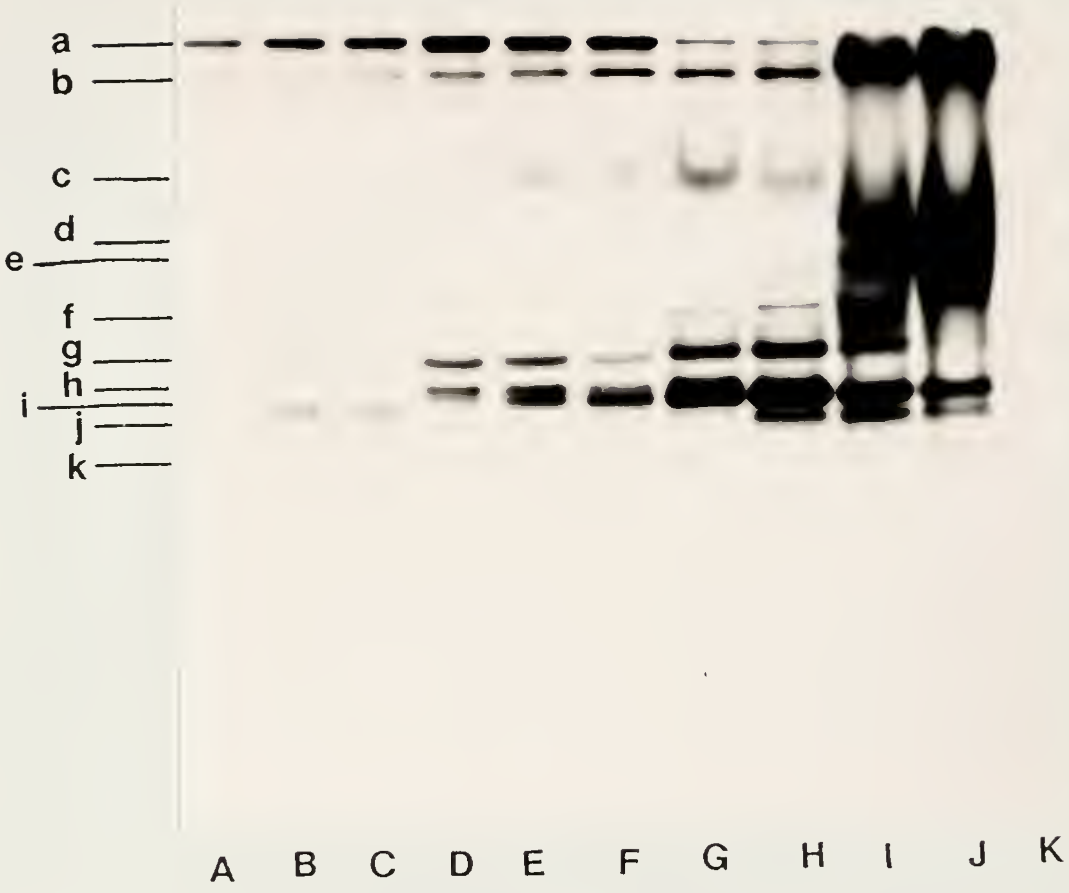

Figure 4-15. PAGE Fluorography of [1,3-^{3}H]DIP-trypsin- and -chymotrypsin-like isozymes of blood fed females of Lu. anthophora. Each lane contained 5 midgut equivalents at different times after blood feeding. The X-ray film was exposed to the gel for 5 days at -70°C. (A) 0 hrs (7,908 cpm), (B) 2 hrs (8,530 cpm), (C) 4 hrs (9,743 cpm), (D) 12 hrs (11,613 cpm), (E) 18 hrs (13,453 cpm), (F) 24 hrs (13,157 cpm), (G) 30 hrs (27,767 cpm), (H) 38 hrs (40,930 cpm), (I) 48 hrs (5,583 cpm), (J) 72 hrs (60,705 cpm) and (K) 96 hrs (4,075).

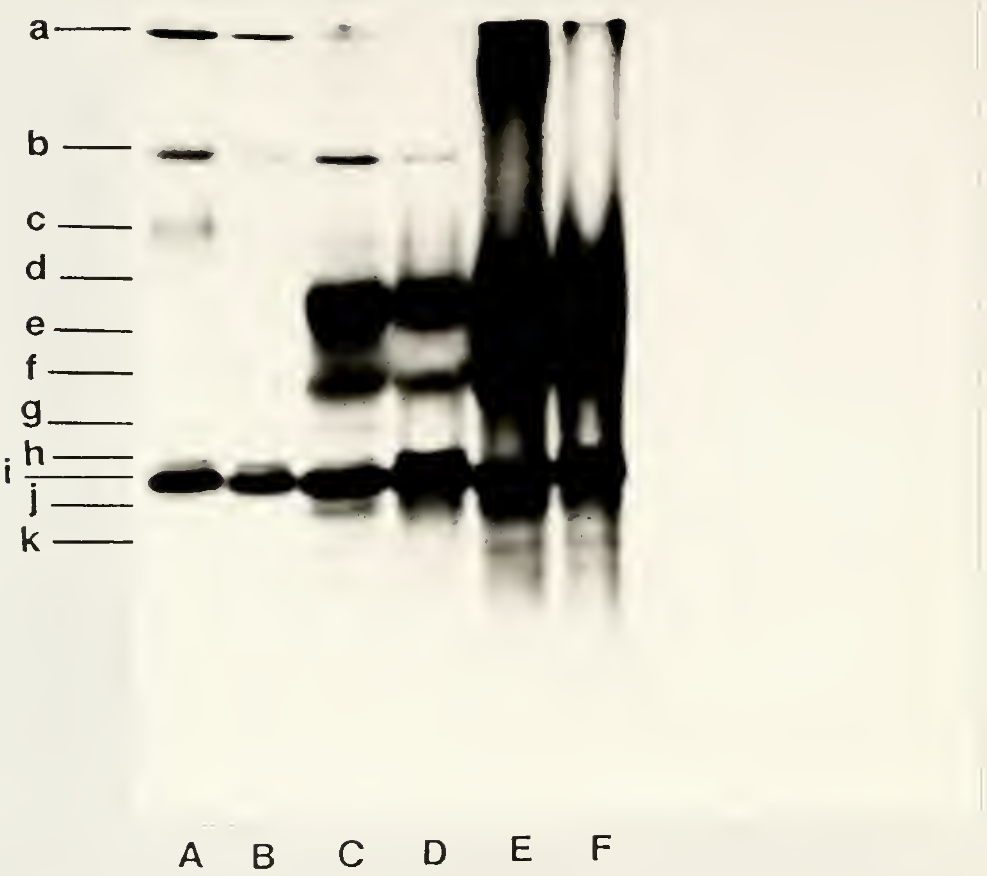

Figure 4-16. PAGE Fluorography of [1,3-^{3}H]DIP-trypsin and -chymotrypsin-like derivatives from blood fed female Lu. anthophora. Each lane contained 9 midgut equivalents. The X-ray film was exposed to the gel for 5 days at -70°C. Lanes A, C and E contained trypsin- and chymotrypsin-like isozymes. Lanes B, D, F and H contained only trypsin-like isozymes. (A) 24 hrs (16,359 cpm), (B) 24 hrs (1,524 cpm), (C) 48 hrs (85,915 cpm), (D) 48 hrs (55,490 cpm), (E) 72 hrs (248,638 cpm) and (F) 72 hrs (96,128 cpm).

Pupae of Lu. anthophora had significantly lower trypsin- and chymotrypsin-like activity compared to 12 to 16-day-old fourth instar larvae (Fig. 4-6). This activity was similar to 5-day-old larvae but their isozymes migrated differently on PAGE (Table 4-2, Fig. 4-10, lanes G & H and Fig. 4-13, lanes A-D). One-day-old pupae had 7 minor bands (a, b, c, g, h, i & m). Only one band "i" was chymotrypsin-like (Fig. 4-13, lanes A & B). Late 8-day-old pupae had 4 minor bands (a, e, g, & h). Only one band "e" had any chymotrypsin-like activity (Fig. 4-13, lanes C & D). Most of the enzymatic activity was contributed by bands "g" and "h".

Different isozymes of females

Newly-emerged unfed and 1 to 3-day-old sugar fed females had significantly smaller amount of isozymes compared to third and fourth instar larvae (Table 4-2, Fig. 4-14, lanes B-C & E-H). Lane D of figure 4-14 contained 9 midgut equivalent of 8-day-old pupae and was below the level of detection. Newly emerged females had 4 minor isozymes (a, g, h, & i)(Fig. 4-14, lane E). One-day-old sugar fed females had an additional band "n". Two to 3-day-old sugar fed females have an additional light band "g" (Fig. 4-14, lanes F-H).

A continuous increase in trypsin- and chymotrypsin-like enzymatic activity of midgut was observed after blood feeding (Table 4-3, Fig. 4-15, lanes A-J). Although several isozymes were synthesized between 0-24 hrs after the blood meal, total activity was not significantly different (Table 4-3, Fig. 4-15, lanes A-F). On the other hand, significant increase in the amounts of trypsin and chymotrypsin was observed at 30, 38, and 48-72 hrs compared to 0-24 hrs after feeding (Table 4-3, Fig. 4-15). A significant decrease occurred in enzymatic activity at 96 hrs after a blood meal (Fig. 4-15). Since there was no significant change in trypsin- and chymotrypsin-like activity between 0-24 hrs after blood feeding, no PAGE was run to distinguish between trypsin- and chymotrypsin-like isozymes (Fig. 4-15).

A total of 11 different trypsin- and chymotrypsin-like isozymes were detected in blood fed females (Fig. 4-15). Only two isozymes; a major band "a" and a minor band "b" were present in newly blood fed females (Fig. 4-15, lane A). Females 2 to 4 hrs after the blood meal ingestion synthesized a major band "a" and 3 minor bands (b, g & i), whereas at 12-24 hrs 6 isozymes (a, b, c, g, h, i & j) were synthesized and 2 bands (c & j) were minor (Fig. 4-15, lanes D-F).

At 30 hrs, females synthesized 3 major bands (b, g & h+i) and 3 minor bands (a, c & f). At 38 hrs an additional isozyme (major band "j") was synthesized (Fig. 4-15, lanes G-H). Females that ingested blood 48-hrs earlier exhibited 7 major bands (a+b, d, e, f, g, h+i & j) and a minor band (k) (Fig. 4-15, lane I).

Three major and 2 minor bands (j & K) were present at 72-hrs. It was not easy to differentiate between the different isozymes of the major bands due to overexposure of the X-ray film. The first major band was composed of bands "a+b" and the second major band occupied the area of bands "d+e+f". Band "g" was absent. The third major band was composed of isozymes "h+i" (Fig 4-15, lane J). No bands were detected 96 hrs after blood ingestion. The blood meal was completely digested in these females (Fig. 4-15).

After inhibiting chymotrypsin-like isozymes with TPCK, bands "a" and "b" had both trypsin- and chymotrypsin-like activity at 24- and 48-hrs after ingestion of blood (Fig. 4-16, lanes A-D). At 72-hrs, however, band "a" was composed of only chymotrypsin-like isozyme (Fig. 4-16, lanes E-F). At 48 hrs band "e" contained chymotrypsin-like activity (Fig. 4-16, lanes C-D).

Discussion

Both trypsin- and chymotrypsin-like enzymes are secreted by the midguts of larvae and adults of Lu. anthophora. Serine protease activity of immature Lu. anthophora increased continuously during larval growth and than decreased in 17-day-old larvae. Similar increases were observed during the first three days of larval growth of Ae. aegypti (Graf & Breigal 1982). The amount of total serine proteases decreased significantly just before pupation and small amounts of trypsin- and chymotrypsin-like enzymes were synthesized in the pupae. Similar decline in proteases were observed in *Ae. aegypti* during metamorphosis (Yang & Davies 1971; Graf & Breigal 1982).

Although both trypsin- and chymotrypsin-like activities were present in *Lu. anthophora*, chymotrypsin was most prominant during larval development. These observations are different from earlier observations in many detritus feeding insects such as the crane fly, *T. abdominalis*, in which major enzymatic activity of larval midgut is trypsin-like (Sharma et al. 1984). Similarly, trypsin- and chymotrypsin-like activities were detected in the midguts of detritus feeding 4th instar larvae of *Rhynchosciara americana*, and tryptic activity was considered more important for food digestion by the larvae (Terra et al. 1979). Although an increase was observed in the quantity of secreted trypsin in 1st-4th instar larvae of *Lu. anthophora*, there was no significant change in the ratio of trypsin (%) to other serine proteases in 1st and 3rd instar larvae. No trypsin- and chymotrypsin-like activity was found in the larval food. A very small quantity of trypsin was secreted in 1 to 8-day-old pupae, but a significant higher trypsin ratio (%) was present.

The larval midgut homogenates of *Lu. anthophora* contained 12 different trypsin- and chymotrypsin-like isozymes. More isozymes were observed in 4th instar larvae compared to 1st and 2nd instar larvae. It might be possible that all 12 different isozymes were secreted in 1st and 2nd instar larvae but their quantities were below the level of detection. Out of the 12 isozymes, 5 were chymotrypsin-like in nature and one isozyme "f" had both trypsin- and chymotrypsin-like activities. Similarly, 12 different isozymes were detected in the midgut homogenate of Ae. aegypti larvae (Kunz 1978). Late fourth instar larvae had low level of serine proteases with 5 different isozymes. Only one isozyme was chymotrypsin-like. The pupae had 7 minor bands, one of which has chymotrypsin-like activity. This was correlated to the very high level of trypsin ratio (%) to other serine proteases.

Low level of trypsin- and chymotrypsin-like enzymes were found in newly emerged and 1 to 3-day-old sugar fed females. A new isozyme, band "n" was detected in 1 to 3-day-old sugar fed females. The amount of trypsin- and chymotrypsin-like enzymes was low at 96-hrs after blood feeding and was similar to pre-blood feeding levels in 3-day-old sugar fed females. Similar observations were reported for Ae. aegypti (Graf & Breigal 1982).

Digestion of the blood meal by female Lu. anthophora induced a continuous increase in the amount of serine proteases for 72 hrs after feeding. Similar observations were reported in other haematophagous Diptera such as Culicidae (Champlain & Fisk 1956; Gooding 1972, 1975;

Breigal & Lea 1975; Graf & Breigal 1982, 1986; & Borovsky 1986, 1988), Simulidae (Yang & Davies 1968), Glossinidae (Langley 1967), and Phlebotominae (Borovsky & Schlein 1987).

High trypsin ratio (%) was present just after blood feeding, however, the amount of trypsin was not very high at this stage. A sharp decline in trypsin ratio (%) occurred within 2-hrs after imbibing a blood meal. High trypsin ratio (%) was present in blood fed females compared to that observed in the larvae of Lu. anthophora. The maximum quantities of trypsin- and chymotrypsin-like enzymes were observed 72-hrs after the blood meal.

In several other insects, maximum serine protease activity was found at different times after blood feeding, e.g., in Ph. papatasi (35 hrs), Cx. nigripalpus (35 hrs) and Ae. aegypti (24-30 hrs)(Borovsky & Schlein 1987; Borovsky 1986; Graf & Briegal 1982; Borovsky 1988). The trypsin ratio (%) declined at 72 hrs after blood feeding and chymotrypsin-like activity was involved in blood digestion at this stage.

A total of 11 different isozymes were detected in blood fed females, and 4 isozymes represented by minor bands (b & C) and major bands (a & e) had chymotrypsin-like activity.

In conclusion, both trypsin- and chymotrypsin-like enzymes were synthesized by the midgut in immature and adult Lu. anthophora. Chymotrypsin-like enzymes are more active during larval development and trypsin-like enzymes are more active in pupae and blood fed females, except for females that took a blood meal 72 hrs earlier.

CHAPTER 5
IN VITRO BIOSYNTHESIS OF JUVENILE HORMONE III BY THE CORPORA ALLATA OF *LUTZOMYIA ANTHOPHORA*

Introduction

Juvenile hormone (JH) plays a major role in immature development and adult reproduction of insects. In mosquitoes it controls mating behavior (Lea 1968; Gwadz et al. 1971), host seeking behavior (Meola & Petralia 1980; Meola & Readio 1987), and stimulates previtellogenic growth of follicles of newly emerged females to resting stage (Lea 1963; Gwadz & Spielman 1973; Spielman 1974; Hagedorn et al. 1977; Mobola & Cupp 1978; Meola & Readio 1988).

Juvenile hormone production remains constant in newly emerged and sugar fed female *Cx*. *pipiens* (Rossignol et al. 1981). Whole body extracts of female *Ae*. *aegypti* showed a peak in JH concentration 2 days after emergence that declined gradually in sugar fed females over the next 5 days (Shapiro et al. 1986). Decline in the synthesis of JH may be due to feed back inhibition induced by the ovaries at the resting stage (Rossignol et al. 1981; Shapiro et al. 1986).

Juvenile hormone stimulates the ovaries to become responsive to the egg development neurosecretory hormone (EDNH) (Hagedorn et al. 1975; Shapiro & Hagedorn 1982) and makes the fat bodies competent to 20-OH-ecdysone. Presence of JH together with ecdysone is essential during vitellogenesis (Borovsky et al. 1985).

Juvenile hormone induces changes in the follicular epithelium of blood fed females and thus is involved in the uptake of vitellogenin (Roth & Porter 1964; Pratt & Davey 1972). Levels of JH III decline sharply after blood feeding (Rossignol et al. 1981; Shapiro et al. 1986; Meola & Readio 1988; Borovsky et al. 1989). In *Ae. aegypti*, JH III level declines within three hours after blood feeding, but is followed by a significant increase until it attains a plateau 46-96-hrs after blood feeding (Shapiro et al. 1986; Borovsky et al. 1989).

In *Cx. pipiens*, secondary follicles of blood fed females require the presence of JH for their growth (Meola & Readio 1988). Juvenile hormone is also required after each gonotrophic cycle to initiate biting behavior (Meola & Readio 1988). Egg retention beyond normal gonotrophic cycle results in the loss of the ability of corpora allata to produce JH. This effect is reversed as soon as females oviposit (Readio et al. 1988).

No information is available about the production of JH in phlebotomine sand flies. Lack of information is due to their small size and small corpora allata which makes it difficult to surgically manipulate these insects. Recently, microtissue culture technique for corpora allata of

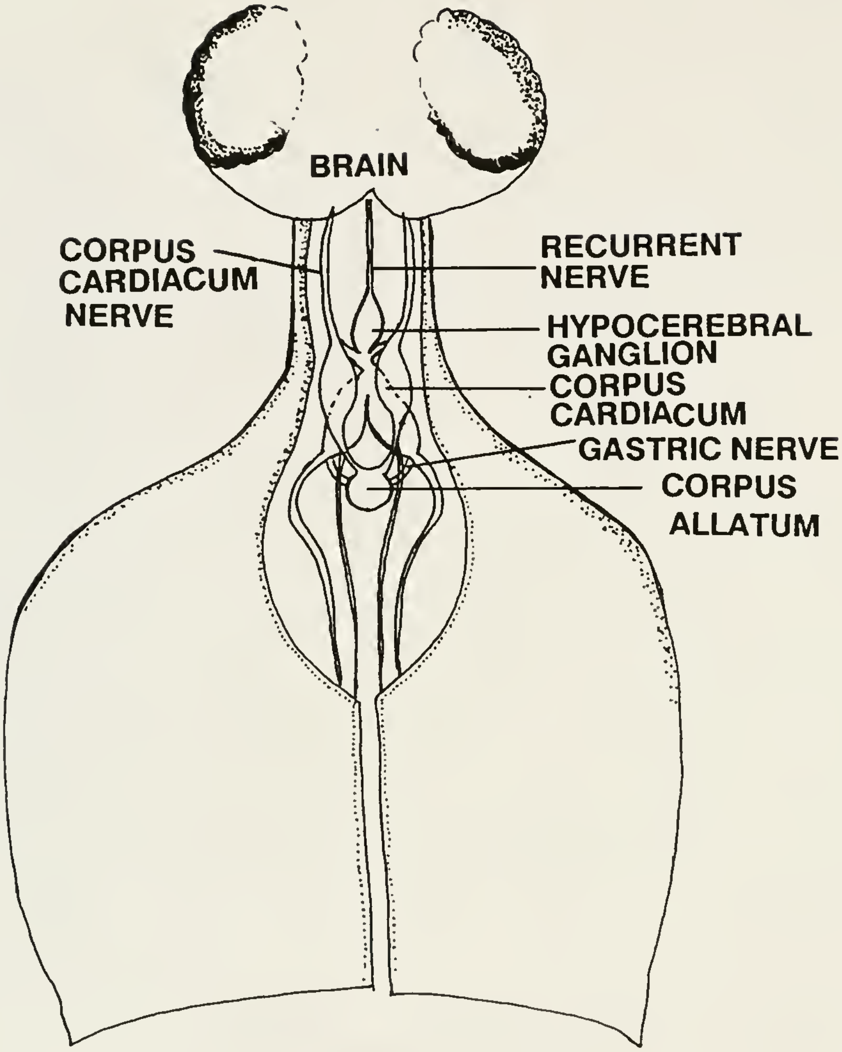

Figure 5-1. Diagrammatic illustration of dorsal view of stomatogastric nervous system and associated endocrine gland in phlebotomine sand flies.

mosquitoes (Borovsky unpublished) has made it possible to determine the in vitro synthesis of JH in sand flies.

The present study describes in vitro biosynthesis of JH by the corpora allata of newly emerged, sugar fed and blood fed females.

Materials and Methods

Strains. Lutzomyia anthophora was maintained under similar environmental conditions in a bioroom as discussed in chapter 3.

Determination of Juvenile Hormone in Adult Lu. anthophora

Juvenile hormone synthesis was determined in the corpora allata of female Lu. anthophora at different times after emergence and blood feeding. The following age groups were used: newly emerged unfed, 12-hrs old unfed, 1-day, 2-days and 3-day-old sugar fed and 3-day-old blood fed females. The blood fed females were dissected 0, 2, 4, 12, 18, 24, 30, 38, 48, 72 and 96 hrs after blood feeding.

Tissue culture of the corpora allata

Juvenile hormone is secreted by the corpora allata, a pair of fused glands situated in the thorax below the junction of the neck and the thorax (Fig. 5-1). Tissue cultures of corpora allata were conducted under sterile conditions in a laminar flow hood. Sand flies were lightly anesthetized with ether and their wings, abdomen and legs

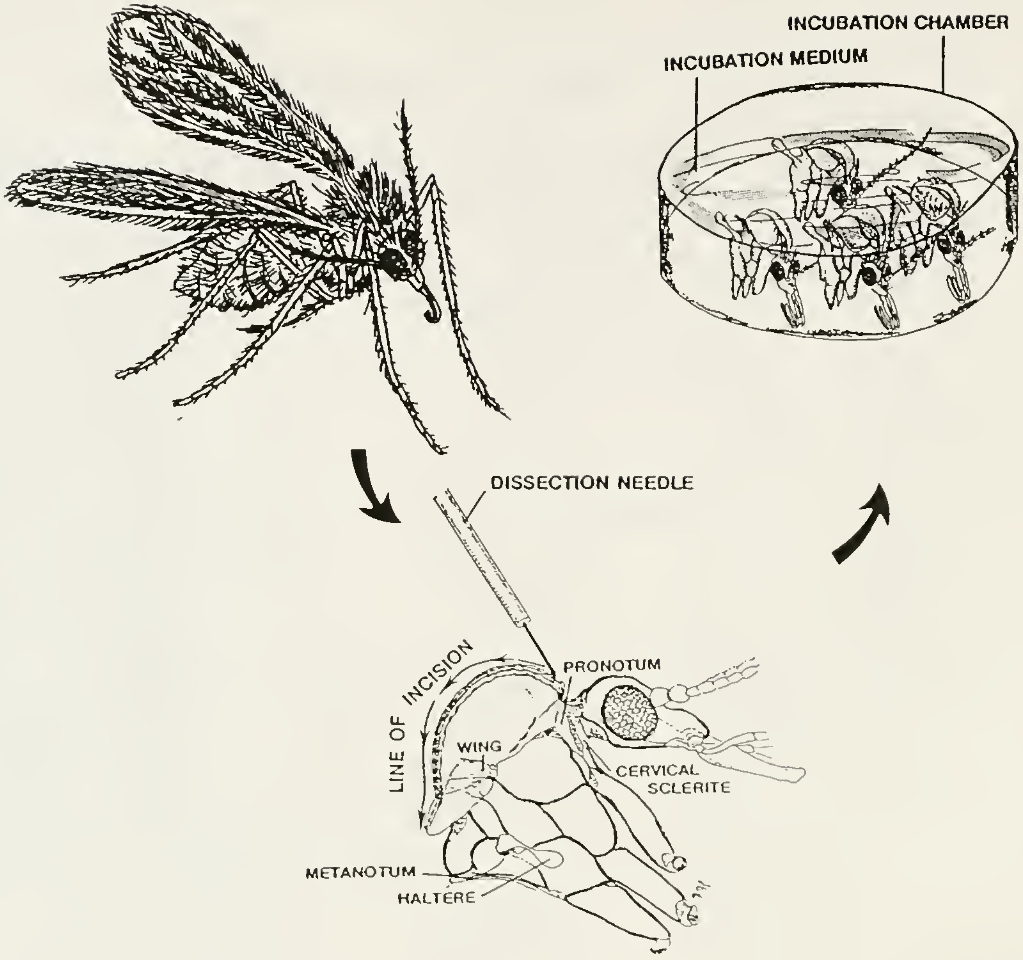

Figure 5-2. In vitro incubation of head-thorax complex of Lutzomyia anthophora for the biosynthesis of JH III. A = female; B = microsurgery of thorax for exposing the corpora allata; C = incubation of head-thorax complexes.

were removed from the head-thorax complex. The intact head-thorax complex was laid with its dorsal side up in a drop of sterile tissue culture medium (TC 199, GIBCO). A fine incision was made on the dorsal side of the thorax from the base of the neck to the end of metathorax with a dissecting needle (Fig. 5-2). The incision was made only in the cuticular layers. The two ends of the cut cuticle were split open with the help of dissection needles. The head-thorax complex was then transferred into a sterile microculture plate coated with silicon (Sigma coat)(Fig. 5-2).

Each culture plate contained 100 µl of sterile tissue culture medium (TC 199, GIBCO) and [12-^{3}H]methyl farnesoate (1 µCi) dissolved in 5 µl of acetone. The [12-^{3}H]methyl farnesoate was provided by Dr. G. D. Prestwich and was prepared as described by Baker and Schooley (1985). Each culture plate contained 20 corpora allata.

Maximum incubation time of corpora allata was determined by incubating 5 samples of 20 corpora allata of females 48-hrs after blood feeding at 27°C. The corpora allata were incubated for 15 minutes, 1, 2, 3 and 4 hrs. Maximum amount of JH III was synthesized after 4 hrs of incubation (See results). Thus, for the remaining experiments, corpora allata were incubated for 4 hrs at 27°C.

After incubation, tissues, culture media, and washings of the culture plates were transferred to a microcentrifuge tube containing 1 ml of acetonitrile. The tissues were homogenized with a teflon tissue grinder and homogenates centrifuged at 14,000g for 20 minutes at 4°C. Supernatants were transferred into another tube and aliquots (20 µl) of the supernatants were added into a tube containing 3 ml of Scintiverse^{tm}. Total radioactivity of this sample was determined in a liquid scintillation system (Tracor analytical Model 6892). To the rest of the supernatant, JH III and JH II (10 µg each) were added, and the volume reduced with a gentle stream of nitrogen to about 50 µl.

Reversed phase C_{18} high pressure liquid chromatography (HPLC) analysis

A standard was run by using [12-^{3}H]-(10_R_)-JH III (2 µl, 400,000 cpm), JH III (10 µg), and JH I (10 µg). The [12-^{3}H]-(10_R_)-JH III standard eluted later than the JH III standard due to increased lipophilicity of trimethyl groups (Hamnett et al. 1981)(Fig. 5-3). To determine the newly synthesized [12-^{3}H]-(10_R_)-JH III by the corpora allata, samples containing JH III, JH II (10 µg each) standards, and newly synthesized [12-^{3}H]-(10_R_)-JH III were chromatographed on 5 µm Microsorb C_{18} reversed phase HPLC column (100 angstrom pore diameter; 4.6mm x 25 cm) using linear gradient of acetonitrile and water (40% to 100%) at a speed of 1.0 ml/min. Juvenile hormone peaks were detected

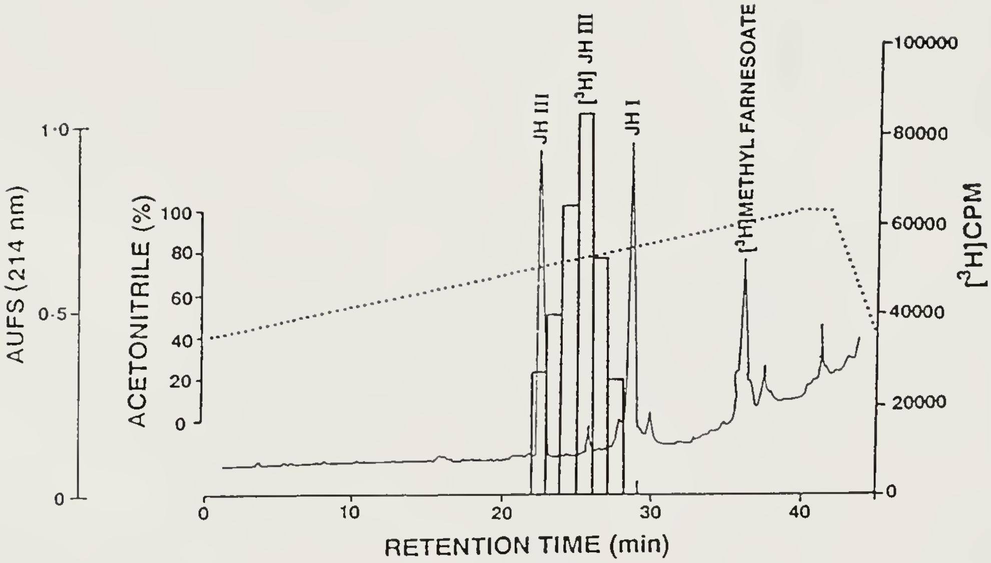

Figure 5-3. C_{18} Reversed phase HPLC of JH III, $[12\text{-}^{3}H]$JH III, $[12\text{-}^{3}H]$methyl farnesoate and JH I. Hormones were chromatographed at a speed of 1.0 ml/min using linear gradient of acetonitrile and water (40%-100%). The chromatography was performed for 40 minutes and fractions (1.0 ml) were collected. Absorbance was followed at 214 nm and radioactivity of samples was measured in a liquid scintillation counter.

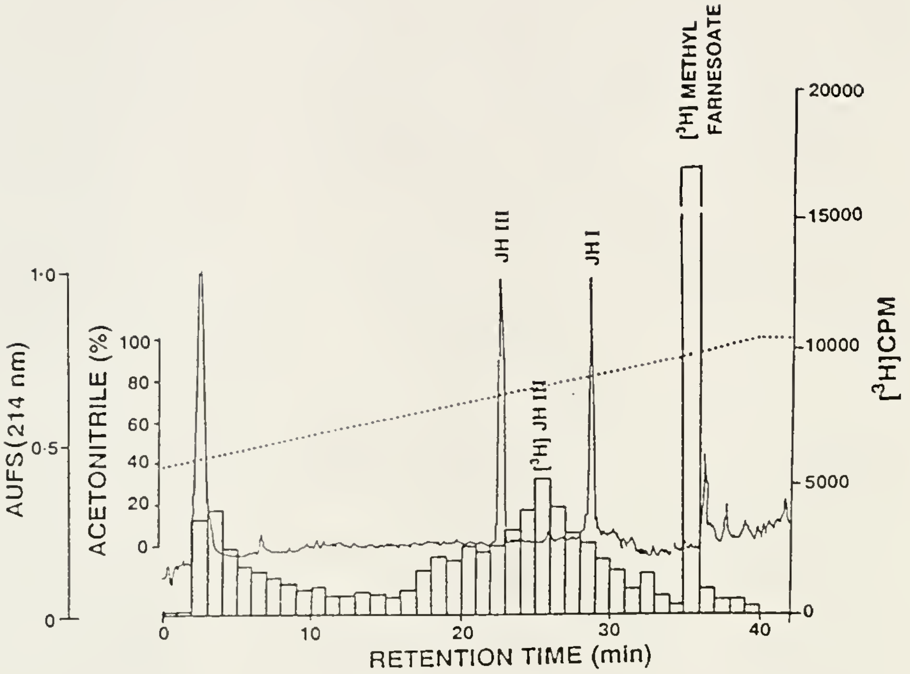

Figure 5-4. A typical example of C_{18} Reversed phase HPLC of [12-^{3}H]JH III, JH III, JH I and [12-^{3}H]methyl farnesoate. Corpora allata of female Lu. anthophora 12-hrs after blood meal were incubated with [12-^{3}H]methyl farnesoate for 4 hrs. Bars represent radioactivity of each 1.0 ml fraction collected during 40 minutes run, using a linear gradient of acetonitrile water (40%-100%). Absorbance was followed at 214 nm.

by UV light at 214 nm. Fractions (1.0 ml) were collected using a fraction collector. Aliquots (200 µl) of each fraction were added to 3 ml of scintiverse^{tm} (Fisher Scientific) and analyzed for radioactivity in a liquid scintillation counter (Fig. 5-3). Thus, fractions which eluted between JH III and JH I markers were collected for gas chromatography studies. Juvenile hormone III standard eluted at 22.82 minutes and JH I eluted 28.84 minutes (Fig. 5-4).

Preparative gas chromatography analysis

Fractions collected for the determination of [12-^{3}H]-(10R)-JH III were concentrated in four groups consisting of 0-3-day sugar fed, 0-24-hr blood fed, 30-48-hr blood fed and 72-96-hrs blood fed females. Gas chromatography was run in D. A. Carlson's laboratory (USDA Insect Infecting Man and Animals Research Laboratory, Gainesville, Florida).

Aliquots from HPLC containing [12-^{3}H]-JH III were collected, reduced under N_2, saturated with NaCl (2g) and extracted 4 times with hexane. The final volume was reduced under N_2 to 15 µl. Samples (15 µl) were injected into a Varian model 2100 preparative gas chromatograph with a flame ionization detector, an integrator, and 1.0 m x 4 mm internal diameter glass column packed with OV-1. Samples were separated with helium gas at 30 ml/min at 160°C isothermal with split ratio of 4:1. Under the above conditions [12-^{3}H]-(10R)-JH III eluted at 2.7 min. Samples

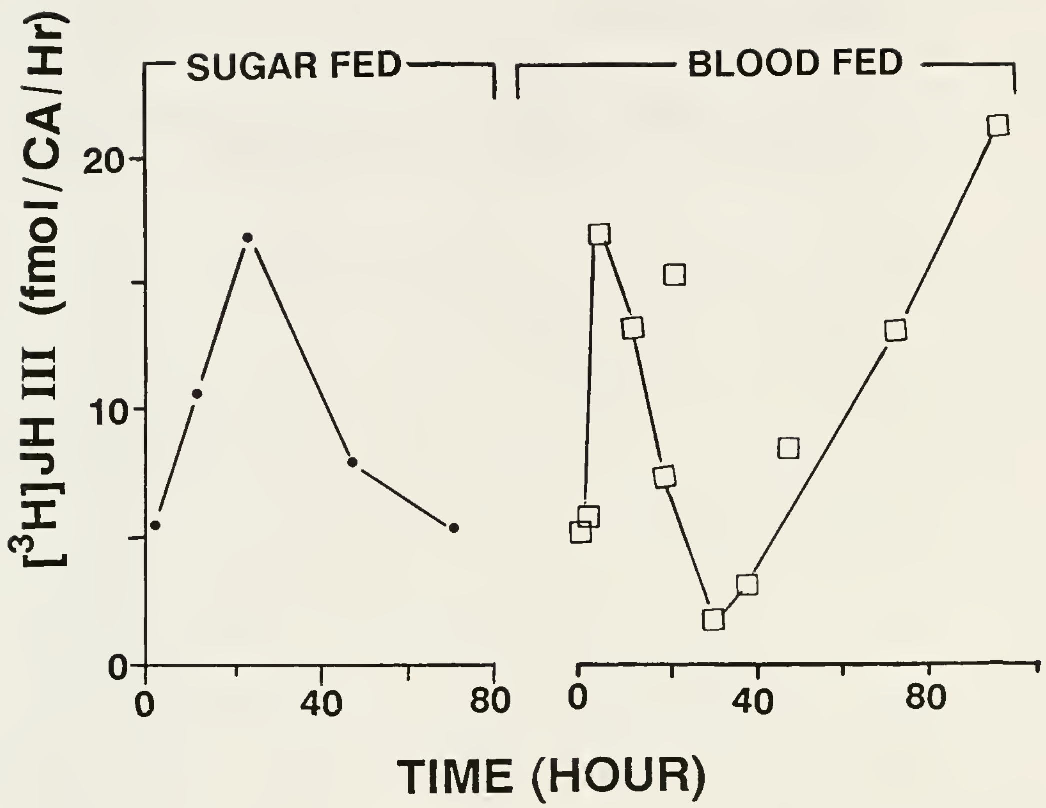

Figure 5-5. Time course of in vitro biosynthesis of [12-^{3}H]JH III in the life of female *Lutzomyia anthophora*. The rate (fm/CA/hr) of [12-^{3}H]JH III biosynthesized by the corpora allata of newly emerged, 12-hrs unfed, 1-day-, 2-day-, 3-day-old sugar fed females (●———●) and 3-day-old blood fed females (□———□), 0-, 2-, 4-, 12-, 18-, 24-, 30-, 38-, 72-, and 36-hrs after blood meal is shown.

were collected in 1.2 x 250 mm gas capillaries at 1 min intervals following JH III, JH II and JHI standard peaks. The JH III peak was collected and washed from the capillaries into instaflour liquid scintillation, and counted in liquid scintillation counter.

Results

Juvenile hormone III and JH I standard peaks eluted at 22.82 and 28.84 minutes respectively (Fig. 5-3). Thus, [12-^{3}H]-(10R)-JH III was collected during 23-29 minutes after the injection of the material in the column (Fig. 5-3). To determine whether the increase in [12-^{3}H]-(10R)-JH III synthesized by corpora allata (CA) was linear, head-thorax complexes were incubated 48-hrs after the blood meal for 15 minutes, 1, 2, 3 and 4 hrs. At the end of incubation [12-^{3}H]-10R-JH III was separated on HPLC but not subjected to gas chromatography.

The amount of [12-^{3}H]-(10R)-JH III synthesized was directly related to the duration of incubation. Thus the rate of [12-^{3}H]-(10R)-JH III synthesized was 8.97 fmol/CA/15 min, 59.66 fmol/CA/1 hr, 72.07 fmol/CA/2 hrs, 97.75 fmol/CA/3 hrs and 269.87 fmol/CA/4 hrs.

The rate of [12-^{3}H]-(10R)-JH III biosynthesis increased in unfed and sugar fed females during 0-24 hrs after emergence (Fig. 5-5). The corpora allata of newly emerged females synthesized 5.63 fmol/CA/hr of [12-^{3}H]-

(10R)-JH III. The rate of biosynthesis increased in 12-hrs old unfed females (Fig. 5-5). The maximum amount of [12-^{3}H]-(10R)-JH III (16.92 fmol/CA/hr) was biosynthesized by 1-day-old sugar fed females . Later, the rate of JH synthesis declined and 3-day-old sugar fed females synthesized only 5.4 fmol/CA/hr of [12-^{3}H]-(10R)-JH III (Fig. 5-5).

In blood fed females a non significant decline occurred in the rate of JH III biosynthesis immediately after blood feeding (Fig. 5-5). The rate of JH biosynthesis started to increase 2-hrs after the blood meal and peaked (17.3 fmol/CA/hr) 4-hrs after blood ingestion. The corpora allata from females 12-hrs after blood feeding synthesized a lesser amount of [12-^{3}H]-(10R)-JH III; the minimum amount (1.6 fmol/CA/hr) was synthesized 30-hrs post blood feeding. The rate of JH III biosynthesis increased 38-hrs after blood feeding and reached the highest levels (21.56 fmol/CA/hr) 96-hrs post blood feeding (Fig. 5-5).

Discussion

Juvenile hormone III has been found in both autogenous mosquitoes, (Guilvard et al. 1984) and anautogenous mosquitoes (Shapiro et al. 1986; Borovsky et al. 1985, 1989; Readio et al. 1988). In vitro JH III biosynthesis by female Lu. anthophora clearly followed a pattern similar to JH biosynthesis in female Ae. aegypti and Cx. pipiens (Shapiro et al. 1986; Borovsky et al. 1989; Meola & Readio 1988). In sugar fed *Ae*. *aegypti* females an increase in JH concentration occurs 2-days after emergence. This is followed by a decline in the amount of JH in the body extract of 5-day-old sugar fed females (Shapiro et al. 1986).

In *Lu*. *anthophora* the rate of [12-^{3}H]-(10*R*)-JH III biosynthesis increased after emergence, with the maximum rate (16.92 fmol/CA/hr) observed in 24-hr-old females. After the peak activity, the rate declined to 5.4 fmol/CA/hr in 3-day-old sugar fed females. A similar decline during *in vivo* JH biosynthesis was noted earlier in sugar fed female *Ae*. *aegypti* and was attributed to feed back inhibition by the resting stage ovaries (Rossignol et al. 1981; Shapiro et al. 1986).

In contrast, no change was observed in the rate of JH biosynthesis in newly emerged and sugar fed females of *Cx*. *pipiens*. The corpora allata of 1 to 8-day-old sugar fed females, incubated with methionine synthesized 18-35 fmol/CA pair/hr of JH III (Readio et al. 1988). *Lutzomyia anthophora* synthesized only 5.4-16.9 fmol/CA/hr of [12-^{3}H]-(10*R*)-JH III. Smaller amounts of JH synthesized by *Lu*. *anthophora* may be contributed to their small size.

After completion of each gonotrophic cycle a new burst of JH is required for the initiation of biting behavior in *Cx*. *pipiens* (Meola & Petralia 1980; Meola & Readio 1987). Similarly, a new supply of JH is required to initiate development of secondary follicles and vitellogenesis after blood feeding ((Readio & Meola 1985).

A rapid decline in the rate of JH biosynthesis occurred in Cx. pipiens and Ae. aegypti within 3-4 hours after blood feeding (Shapiro et al. 1986; Readio & Meola 1988; Borovsky et al. 1989). No such decline in the rate of synthesis of [12-^{3}H]-(10R)-JH III was observed in Lu. anthophora within the first few hours. The rate in 3-day-old females immediately after blood feeding was similar to that of 3-day-old sugar fed females. The rate of JH III biosynthesis increased 2 to 4-hrs after the blood meal; after 4 hrs it was similar to the rate of biosynthesis in 24-hr-old sugar fed females. This rise in JH III production may be necessary to enhance the effect of 20-OH-ecdysone during vitellogenesis (Borovsky et al. 1985; Racioppi et al. 1986).

In Lu. anthophora the rate of [12-^{3}H]-(10R)-JH III biosynthesis declined 12-hrs after blood feeding, reaching a minimum (1.57 fmol/CA/hr) at 30-hrs. This amount was lower than that synthesized by 3-day-old sugar fed and recently blood fed females. In Ae. aegypti a sharp decline in the amount of JH III was observed 3-hrs after the blood meal but the rate of biosynthesis increased 8 to 48-hrs after the blood meal (Borovsky et al. 1989). The amount of JH III was similar in sugar fed females and females that were blood fed 96-hrs earlier (Shapiro et al. 1986). Similarly, the rate of JH III biosynthesis in Cx. pipiens declined after blood feeding (Readio et al. 1988). The rate increased between 72 to 96-hrs after the blood meal and was similar to sugar fed females (Readio & Meola 1988). The rate of [12-^{3}H]-(10R)-JH III production increased 38-hrs post blood meal in *Lu. anthophora* and reached a maximum level 96-hrs after the blood meal. The rate of synthesis in females 96-hrs after blood feeding was much higher than that of sugar fed females.

Juvenile hormone III biosynthesis by *Lu. anthophora* is similar to mosquitoes. It is tempting to speculate that perhaps vitellogenesis in sand flies may be regulated by both 20-OH-ecdysone and JH III as was shown for female *Ae. aegypti* (Borovsky et al. 1985).

CHAPTER 6
REPRODUCTIVE STATUS AND AGE STRUCTURE OF SOME COLOMBIAN PHLEBOTOMINE SAND FLIES

Introduction

The epidemiology of a vector borne disease in a human population depends on many factors linked to the vector, reservoir, and parasite. Some of these, regarding the vector, include longevity, anthropophily, genetic susceptibility to a parasite and proportion of population that is infected in nature. Circadian biting behavior of the infected vector, related to human or animal reservoir activity patterns, determines the rate of transmission. A harmony between the longevity of vector and extrinsic development of the parasite determines the number of infected insects found in an area. The survival of an infected vector compared to a noninfected vector depends on the pathogenicity of the parasite to the vector.

Time required for the extrinsic development of the parasite in the vector depends largely on temperature of the vector's micro habitat (Leany 1977; Killick-Kendrick 1979). For example, at high temperatures a smaller number of sand flies develop Leishmania infection because anterior migration of the parasite is inhibited (Leany 1977). The presence of primary and secondary reservoirs of the disease in the area, socioeconomic level of the human population at risk and the health facilities are also important in the demography of the diseases.

Most of the above factors are not known in full detail for Leishmania transmission in Colombia. In the present study, an attempt was made to determine the longevity of some Lutzomyia sand flies in Northeastern Colombia where active transmission of cutaneous leishmaniasis occurs.

Materials and Methods

Rate of Egg Development in Lu. anthophora

Because laboratory colonies of Colombian sand flies were not available, Lu. anthophora, a sand fly from Mexico and the U.S.A., was used to determine the applicability of the age grading technique of Detinova (1962). Colony-bred Lu. anthophora were maintained as discussed in Chapter 3. Three-day-old Lu. anthophora were blood fed on a hamster for one hour; their ovaries were dissected after 24, 48, 72, and 96 hrs following blood feeding. Three to ten females were dissected at each interval; the length and width of their ovarian follicles and length of the ovarioles occupied by yolk were measured at 10X with an ocular micrometer under a compound microscope. Measurements were made from 5-10

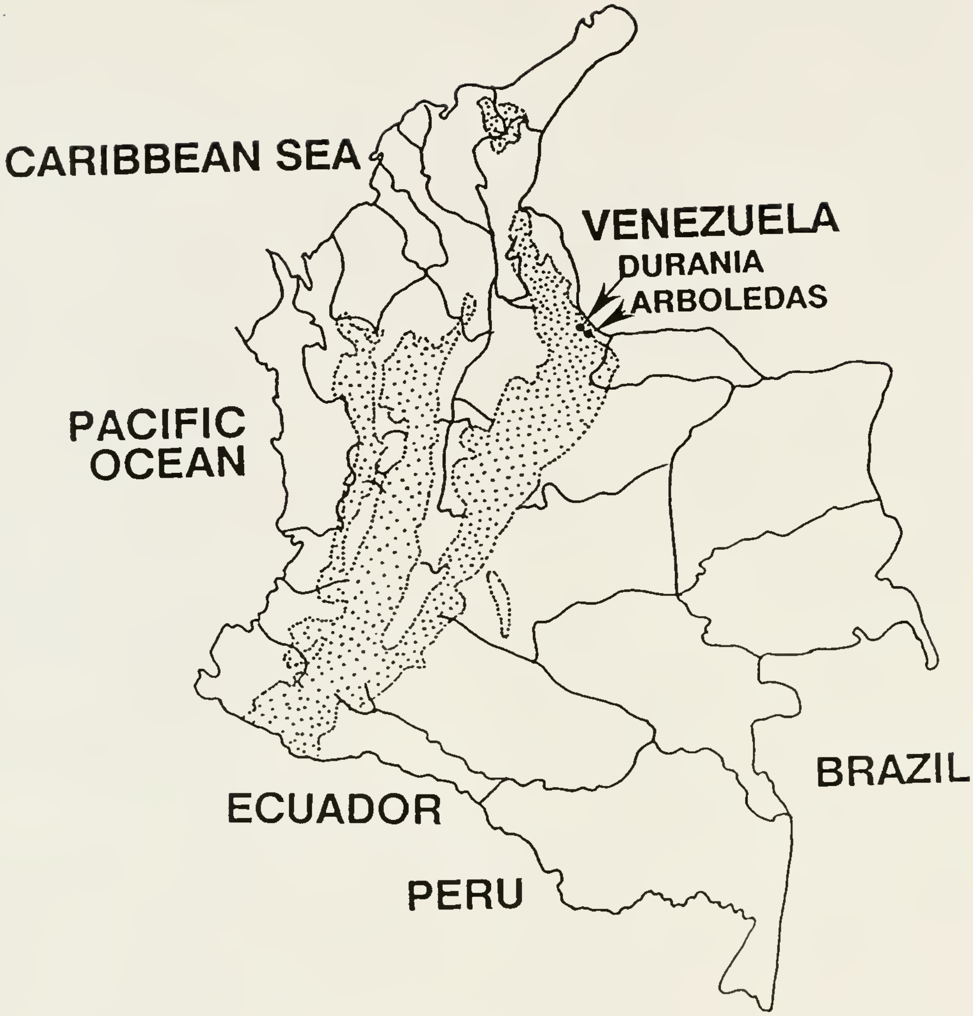

Figure 6-1. Map of Colombia showing location of Arboledas and Durania.

ovarioles from each female. Measurements of ovarioles were also made for comparison from newly emerged females, 3-day-old sugar fed females, and of eggs after oviposition.

Description of the study sites

Colombia, an ecologically diverse country, is the northernmost republic in South America (Fig. 6-1). It can be divided into 23 vegetative zones comprising tropical forest to permanent snow covered mountains and desert bush (Holdridge 1947). Unique environmental conditions are created by the Andes mountains that split into three north-south oriented mountain ranges. The eastern, central and western ranges are separated from each other by deep valleys of Cauca and Magdalena (Fig. 6-1). Most of the colombian population is concentrated in these two valleys.

The present study sites of Arboledas and Durania are situated in Norte de Santander Department, in areas of Colombia (7° 39'N, 72° 48'W). The population of Arboledas consists of approximately 19, 000 inhabitants. Five thousand people inhabit the main town; the remainder live in the surrounding country side (Alexander 1988).

During 1985-86, investigations concentrated on sand flies collected from two coffee plantations, La Esperanza and La Quinta in the district of Sirvata, situated 3 Km east of Arboledas. Both plantations are inhabited by people with large families. Their houses are situated closely to shaded coffee plantations.

Vegetation. The vegetation of La Esperanza, described earlier (Alexander 1988), is composed of scattered large trees ('ceibo' *Erythrina* sp. and 'curo canelo' *Nectandra* sp.) that reach heights of 30 m. These trees support strangler 'figs' (*Ficus* sp.) and numerous different epiphytes. Several other small trees are also present; the forest floor is covered with leaf litter and extensive stands of 'balsam'(*Impatience* sp.) (Alexander 1988).

The vegetation of La Quinta is some what different from La Esperanza. The tree trunk resting collection were made from large trees comprised of 'guacharaco'(*Cupania cinera*), 'Pardillo' (*Cordia alliodora*), 'ceibo' (*Erythrina* sp.), 'tarai' (*Simarouba amara*), 'bara blanca'(Unknown species) and 'caracoli' (*Anacardium excelsum*). The most important tree in both areas was 'guamo' (*Ingua edulis*). Other trees are mostly fruit trees such as orange, mango, banana, guava, zapote (*Matisia cordata*) and cacao (*Theobroma cacao*) (Alexander 1988).

The coffee plantations of Durania were situated 20 Km of the village of Durania. Sand fly collections were made at this site from January 1987 to January 1988. The ecological conditions and vegetation are very similar to that of Arboledas, except that rock outcroppings were present near and in coffee plantations (Alexander 1988).

Potential reservoir hosts. Fourteen different mammalian species were discovered in the study areas during

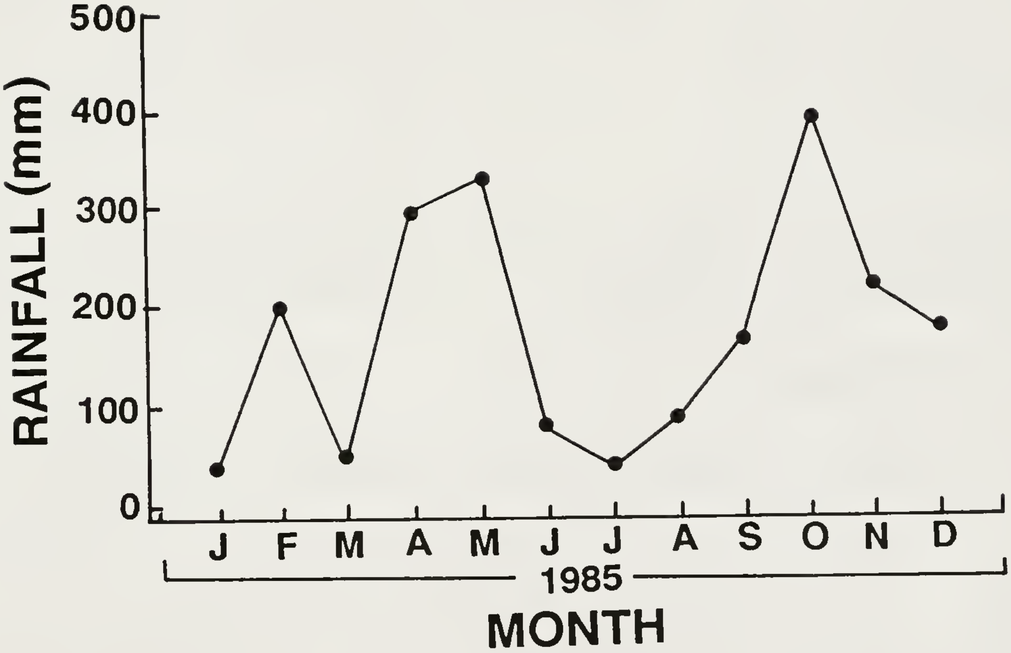

Figure 6-2. Rainfall in Arboledas (Colombia) during 1985.

1985-1986 animal survey (Alexander 1988). The opossums (*Didelphis marsupials*, *Caluromys lanatus*, *Metachirus nudicaudatus*, and *Marmosa* sp.), the rats (*Oryzomys* sp. and *Rattus rattus*), the squirrel *Sciurus granatensis*) and the tree porcupine (*Coendou* sp.) also live in the area. Various domestic animals such as dogs, horses, mules and pigs are owned by the inhabitants.

Rainfall. In the present study sites, the wettest periods of the year were mid-March to mid-June and October to mid-December. Peak rain fall occurred in March-April and October-November (Fig. 6-2).

Sampling Methods

Adult sand flies were collected from Arboledas during May 1985 to September 1986 and from Durania during January 1987 to January 1988. Sand flies were collected by the following 6 different methods.

Tree Trunk Collections

Adults sand flies were collected daily from tree trunks from 9 A. M. to 11 A. M. Two to 10 collectors, depending upon the availability of man power, collected the sand flies for at least one hour by using mouth aspirators. Resting sand flies were disturbed from the tree trunks, tree holes and root buttresses by stroking the surfaces with twigs. The disturbed sand flies were collected when they landed nearby. Sand flies were gently blown from the aspirator into 120 ml specimen containers. Later these were brought to the laboratory where the flies were frozen for future processing (Young et al. 1979).

Human Bait Collections

During these collections, 2 to 10 volunteers sat on the ground with flash lights and captured sand flies that came to suck blood. Sand flies were collected from 1800 to 2100 hrs by using mouth aspirators.

Light Trap Collections

CDC light traps (Sudia & Chamberlain 1966), powered by four 1.4 volts batteries were used throughout the study. Light traps were run over night and the collections were brought to the laboratory for further processing. After separation of the sand flies they were frozen in liquid nitrogen (Young et al. 1979).

Leaf Litter Collections

Sand flies resting in leaf litter were collected by placing a wooden-framed gauze cage (one foot square) over forest floor. Leaf litter was disturbed and sand flies that landed on the gauze were aspirated, transferred to 120 ml specimen containers and brought to the laboratory (Alexander 1988).

Indoor Resting Collections

Sand flies that came to seek a blood meal from the residents of the houses or their domestic animals were collected from inside the houses at night.

Resting Collections from the Rocks

Resting collections were made from near or under the rocks by disturbing the sand flies resting in this habitat. This method was applied only at Durania.

Dissections Methods

Sand flies were identified by excising their spermathecae with fine dissecting needles (insect pins that were inserted in wooden applicators) into a drop of physiological saline and observing them at 40x under a compound microscope. Similarly, the reproductive system of females was excised into a drop of physiological saline, the ovary disrupted as described by Hitchcock (1968) and ovarioles observed under a compound microscope at 100x without a coverslip. Ovarian maturation was classified according to the scheme of Christophers (1911) as modified by Mer (1936). Dilatations were classified according to the scheme of Detinova (1962). Trophic status was classified as unfed, fresh fed and late fed. Unfed females did not have any blood meal in the midgut but they might be sugar fed,

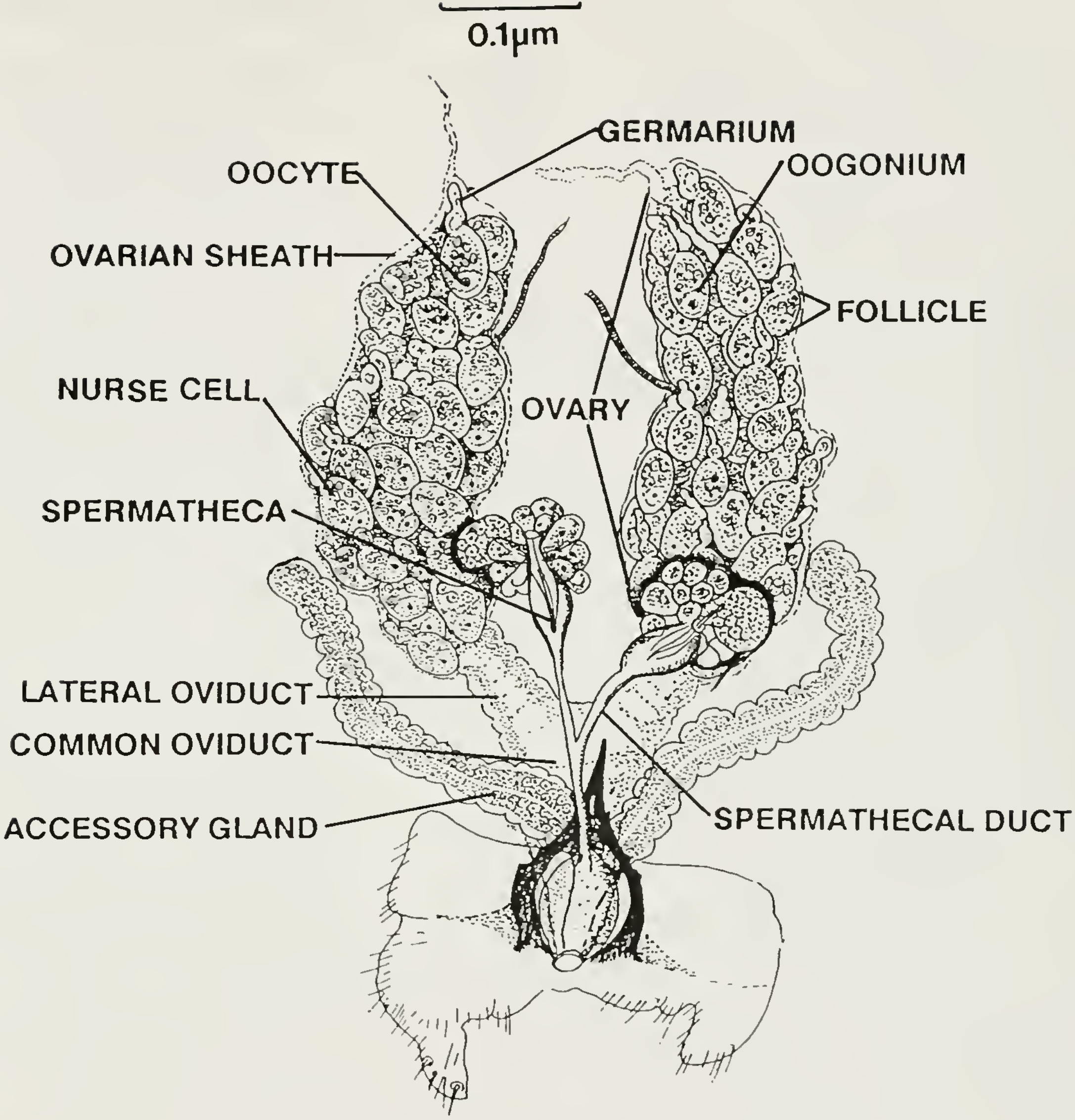

Figure 6-3. Morphology of the female reproductive system of Lu. anthophora.

Late fed females were mostly gravid. Fresh fed females contained fresh blood meals.

Calculations

The proportion of parous females was the total number of parous females in a collection / Total number of nulliparous + parous females in a collection Davidson (1954). No information is available about the duration of gonotrophic cycle of the Colombian species dissected due to lack of laboratory colonies. Therefore, no attempt was made to calculate their survivorship.

Results

Morphology of Female Reproductive System and Rate of Egg Development in Lu. anthophora

The female reproductive system of *Lu. anthophora* consists of a pair of ovaries, each with a large number of ovarian follicles (Fig. 6-3). Each ovary is enveloped in an ovarian sheath. Both ovaries open into lateral oviducts that unite to form a common oviduct. The common oviduct opens to the exterior by the genital opening. A pair of accessory glands also opens here. Two spermathecae are present, each is oblong and possesses a number of evaginations at the top (Fig. 6-3). Each spermatheca opens into a lateral spermathecal duct that unites to its other

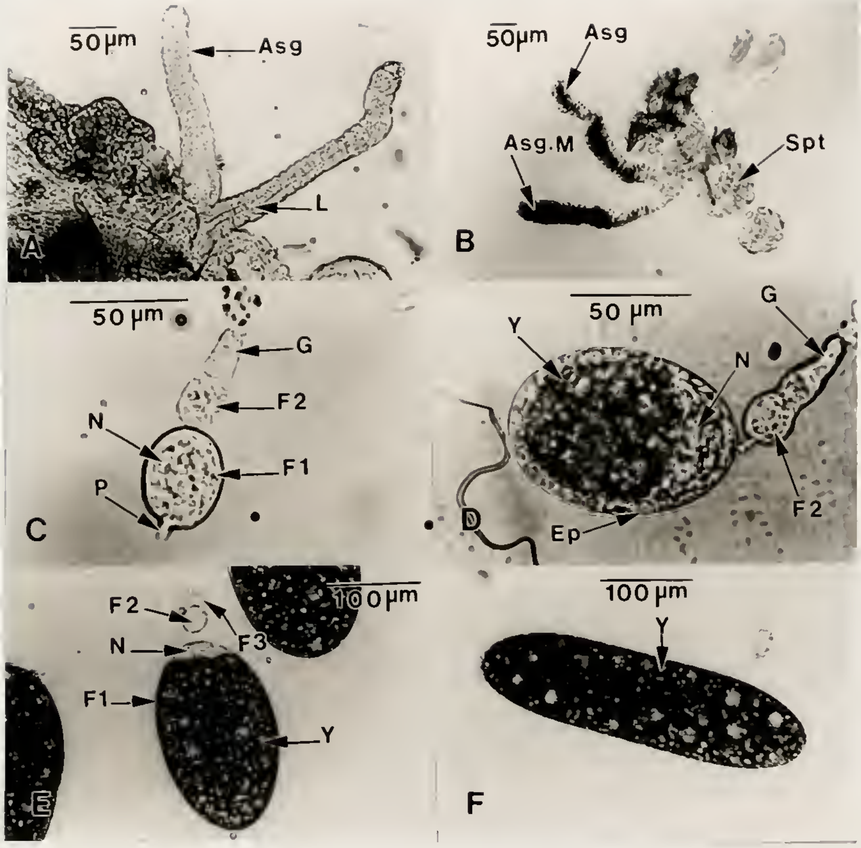

Figure 6-4. Different parts of female reproductive system of Lu. anthophora. A = accessory glands of a nulliparous unfed female; B = accessory glands of a blood fed nulliparous female; First stage (resting follicle) of nulliparous female; D = ovarian follicle of blood fed female 24 hrs after blood feeding (late stage II); ovarian follicle of a blood fed female 48 hrs after blood feeding and F = mature fifth stage egg, 96 hrs after blood feeding. Asg = female accessory gland; Asg. M = accessory gland material; Ep = follicular epithelium; F1, F2 and F3 = primary, secondary, and tertiary ovarian follicles; G = germarium; N = nurse cells; P = pedicle; Spt = spermatheca and Y = yolk granules.

Table 6-1. The rate of egg development in *Lu. anthophora* after blood feeding.

Time (Hours)	Follicle (length)	Follicle (width)	Yolk (length)
	$\overline{\mu m}$ ± SE$\bar{X}$ (N)*	$\overline{\mu m}$ ± SE$\bar{X}$ (N)*	$\overline{\mu m}$ ± SE$\bar{X}$ (N)*
Newly Emerged	38.1 ± .8 (26)^{E}	34.2 ± .4 (25)^{D}	0.0
3-day-old (SF)	44.8 ± .7 (24)^{E}	36.3 ± .5 (24)^{D}	0.0
3-day-old (BF)			
24 hrs	90.5 ± 1.8 (90)^{D}	58.9 ± 1.1 (91)^{C}	61.24 ± 1.6 (89)^{D}
48 hrs	175.8 ± 1.8 (90)^{C}	NE**	156.0 ± 2.1 (100)^{C}
72 hrs	273.1 ± 5.3 (100)^{B}	NE**	273.4 ± 5.3 (100)^{B}
96 hrs	339.1 ± 2.2 (98)^{A}	72.5 ± .9 (98)^{B}	339.1 ± 2.2 (99)^{A}
Oviposition	344.5 ± 1.8 (49)^{A}	78.2 ± .7 (48)^{A}	

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* Mean ± standard error of mean (sample size) followed by same letters were not significantly different when tested by Duncan's new multiple range test ($P < .05$) ($\alpha = .05$). ** Not Estimated. SF = Sugar fed and BF = Blood fed females.

partner to form a common spermathecal duct that opens into a bladder-shaped vagina (Fig. 6-3).

The accessory glands of unfed females are pale and lack a granular substance (Fig. 6-4-A). The accessory glands of blood fed females are opaque, and filled with a granular accessory gland substance that increases in amount as the egg development proceeds (Fig. 6-4-B). Primary egg follicles (F1) of newly emerged females are very small and lack any differentiation into nurse cells and oocyte. After sugar feeding, the primary follicle increases in its length and width, attaining the stage N as described for anophelines by Mer (1936) (Table 6-1). The stage N or resting follicle has 7 monomorphic nurse cells (N) and an oocyte (Fig. 6-4-C). The secondary follicle (F2) is not separated from the primary follicle at this stage (Fig. 6-4-C).

The primary follicle increases significantly in length and width within 24 hrs after blood feeding; 68 % of its length is then occupied by yolk granules. The secondary follicle can be distinguished from the primary follicle at this age (Table 6-1) (Fig. 6-4-D). A significant increase occurred in the length of ovariole and the length of the follicle occupied by the yolk (Table 6-1)(Fig. 6-4-E). Complete ovarian maturation is attained after 96 hrs and the size of the ovarioles is similar to that of the eggs deposited by females at 96-120 hrs after blood feeding

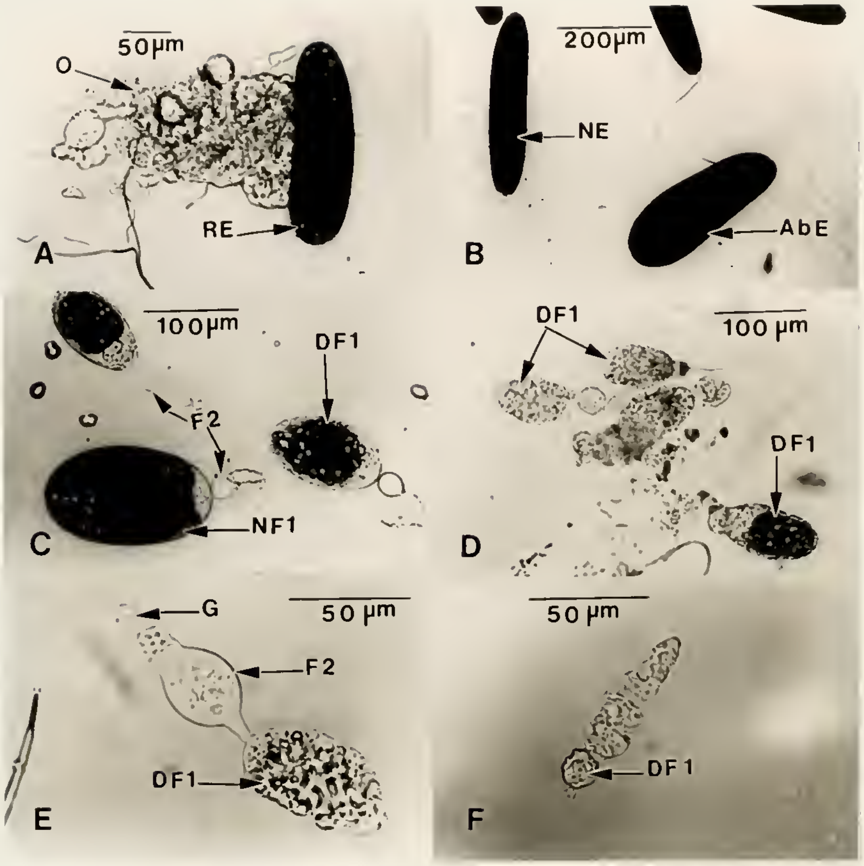


Figure 6-5. Different stages of ovarian follicles of Lu. anthophora. A = relict egg (RE) in ovary of a uniparous female; B = normal fifth stage eggs (NE) and abnormal fifth stage egg (AbE) 96 hrs after blood feeding; C = Normal primary follicle (NF1) and degenerated primary follicle (DF1) 48 hrs after blood feeding; D = two types of degenerated ovarian follicles 48 hrs after blood feeding; E = completely degenerated primary follicle (DF1) of a blood fed female 48 hrs after blood feeding and F = primary degenerated follicle of a sugar fed female (DF1). F2 = secondary follicle; G = germarium; O = ovary.

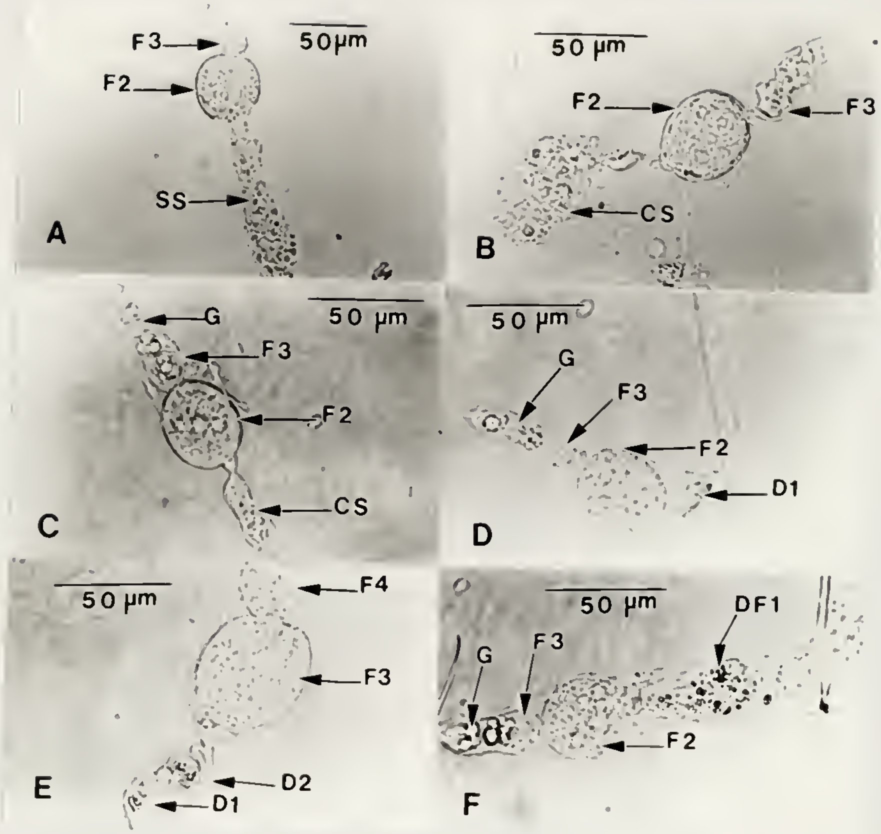


Figure 6-6. Different stages of ovarian dilatation formation in Lu. anthophora. A = Sack stage (SS) seen just after oviposition; B = half contracted sack stage (CS); C = almost completely contracted sack stage (CS); D = Completely formed dilatation (D1) after first oviposition; E = Two dilatations in an ovarian follicle; F = degenerated egg from the ovary of a nulliparous gravid female. D1 = dilatation formed after first oviposition; D2 = dilatation formed after second oviposition; DF1 = primary degenerated follicle; F1, F2, F3 and F4 = primary, secondary, tertiary and quaternary follicles; and G = germarium.

(Table 6-1)(Fig. 6-4-F). Sometimes one to several relict eggs are present in the ovaries of females that oviposited (Fig. 6-5-A). A few slipper-shaped abnormal eggs (Ab E) were found in the ovaries of some females (Fig. 6-5-B). In others, many follicles degenerated 48 hrs after blood feeding (Fig. 6-5-C, 6-5-D & 6-5-F). Such follicles had little yolk or lacked it completely (Fig. 6-5-D & 6-5-E). One sugar fed female showed degeneration of a primary follicle (Fig. 6-5-F).

The follicles of females that had recently oviposited showed sack stage (SS) (Fig. 6-6-A). The secondary follicle (F2) of such females had 7 well differentiated nurse cells and an oocyte. It was difficult to determine the rate of contraction of the sack stage due to poor survival of the females after oviposition. But different stages of contraction of sack (CS) were seen in various females (Figs. 6-6-B,6-6-C & 6-6-D).

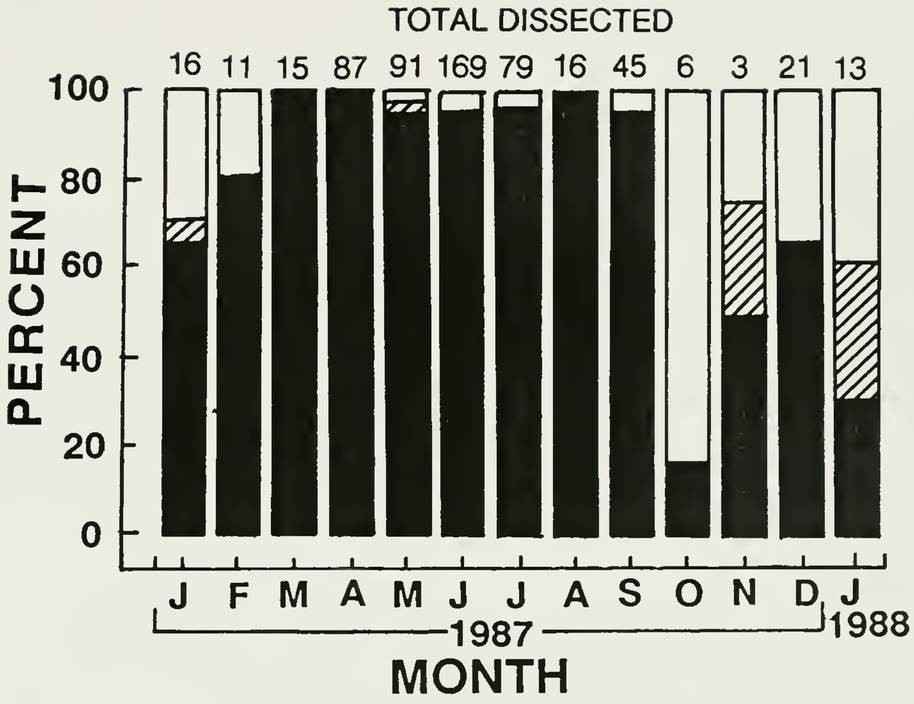
Field collected specimens from Colombia showed 1 to 4 dilatations. Many females had sack stage only and no degenerated eggs were found. Such females were classified as parous. It was possible to age grade some wild caught gravid females by finding degenerated eggs (Fig. 6-6-D). But it was not possible to classify all gravid females and they were classified as "not estimated". Some parous females contained relict eggs. The females that contained

Table 6-2. Phlebotomine sand flies collected at Arboledas and Durania, Colombia.

Species	Arboledas 1985-1986	Durania 1987-1988
Lutzomyia spinicrassa Osorno-Mesa, de Osorno & de Hoyos 1969	*	*
Lutzomyia serrana (Damasceno & Arouck 1949)	*	*
Lutzomyia shannoni (Dayar 1929)	*	*
Lutzomyia ovallesi (Ortiz 1952)	*	*
Lutzomyia gomezi (Nitzulescu 1931)	*	*
Lutzomyia nuneztovari (Ortiz 1954)	*	*
Lutzomyia davisi (Root 1934)	*	*
Lutzomyia walkeri (Newstead 1914)	*	
Lutzomyia dubitans (Sherlock 1962)	*	*
Lutzomyia pia (Fairchild & Hertig 1961)	*	*
Lutzomyia erwindonaldoi (Ortiz 1978)	*	*
Lutzomyia lichyi (Floch & Abonnenc 1950)	*	*
Lutzomyia sauroida Osorno-Mesa, Morales-Alarcon & de Osorno 1972	*	*
Lutzomyia atroclavata (Knab 1913)	*	
Lutzomyia ferroae Young & Morales 1987	*	*
Warileya rotundipennis Fairchild & Hertig 1951	*	*
Brumptomyia beaupertuyi (Ortiz 1954)	*	*
Lutzomyia longipalpis (Lutz & Neiva 1912)		*
Lutzomyia olmeca bicolor (Fairchild & Theodor 1971)		*
Lutzomyia cayennensis (Floch & Abonnence 1948)		*
Lutzomyia trinidadensis (Newstead 1922)		*

Figure 6-7. Temporal changes in the reproductive status of *Lu*. *cayennensis* collected resting on tree trunks in Durania, Colombia during January 1987 to January 1988. ■ Unfed; ▨ fresh fed; □ late fed.

Figure 6-8. Temporal changes in the ovarian development stages of *Lu*. *cayennensis* collected resting on tree trunks in Durania, Colombia during January 1987 to January 1988. ■ First stage; ▧ second stage; ░ third stage; ▩ fourth stage and □ fifth stage of ovarian follicles.


TOTAL DISSECTED
16
11
15
87
91
169
79
16
45
6
3
21
13
100
80
60
40
20
0
PERCENT
J
F
M
A
M
J
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A
S
O
N
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1987
1988
MONTH

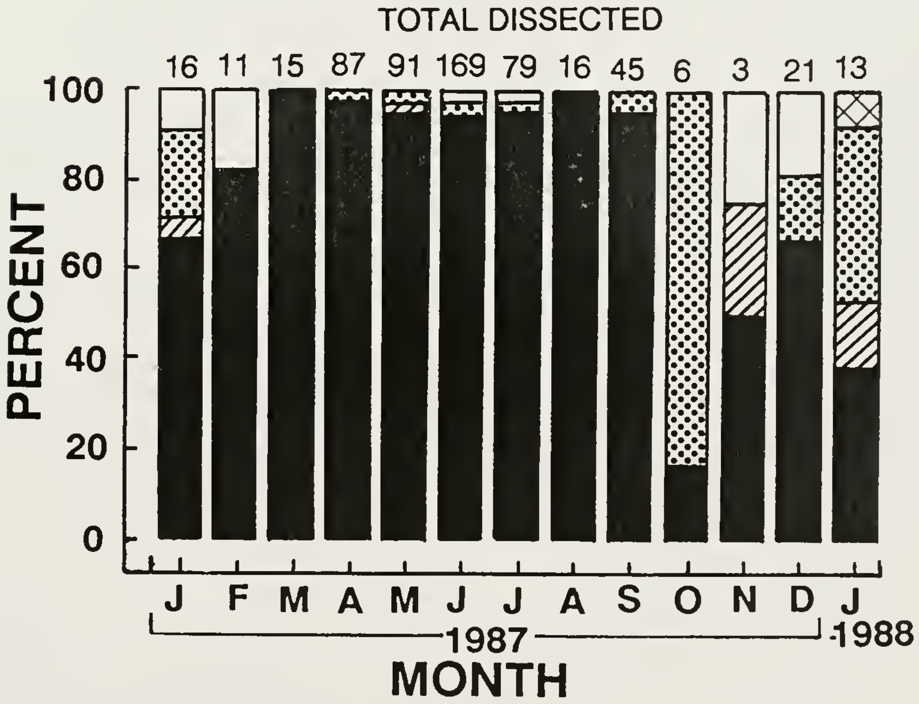

TOTAL DISSECTED
16
11
15
87
91
169
79
16
45
6
3
21
13
100
80
60
40
20
0
PERCENT
J
F
M
A
M
J
J
A
S
O
N
D
J
1987
1988
MONTH

Table 6-3. Changes in age structure of *Lu. cayennensis* collected by various methods in Durania, Colombia during January 1987 to January 1988.

Month	Total* Diss.	P**	Number of Dilatations 0(%)	1(%)	2(%)	3(%)	4(%)	NE***	Sack**** Stage(%)
Tree Trunk Resting									
January	21	0(.00)	20(100)						
February	11	0(.00)	11(100)						
March	15	2(.13)	13(87)	1(7)	1(7)				
April	87	6(.07)	80(93)	1(1)				1	5(6)
May	91	7(.09)	82(92)	3(3)	2(2)			2	2(2)
June	169	15(.09)	149(91)	2(1)	4(2)	1(1)		5	11(7)
July	79	15(.19)	64(81)	6(8)	3(4)			3	6(8)
August	16	1(.06)	15(94)				1(7)		
September	46	6(.15)	35(85)	4(10)				5	2(5)
October	6	0(.00)	6(100)						
November	4	1(.50)	1(50)					2	1(25)
December	21	7(.33)	9(43)	6(29)	1(5)			5	
January	13	5(.38)	8(61)	5(38)					
Human Bait									
April	6	2(.33)	4(67)		2(33)				
August	5	0(.00)	3(100)					2	
Leaf Litter									
August	15	1(.06)	14(93)		1(7)				
September	4	2(.50)	2(50)						2(50)

Table 6-3--extended.

Month	Total* Diss.	P(porp.)**	Number of Dilatations						Sack**** Stage(%)
			0(%)	1(%)	2(%)	3(%)	4(%)	NE***	
			Indoor Resting						
May	5	0(.25)	3(75)		1(25)			1	
June	2	0(.00)	2(100)						
July	6	1(.17)	5(83)	1(17)					
August	2	0(.00)	2(100)						
			Rocks						
December	19	1(.06)	15(83)	1(6)					1(6)

*Total females dissected each month. ** Parous females (proportion parous i.e, parous/nulliparous+parous). *** Not estimated; it was not possible to find any dilatations. **** Females that have recently oviposited. It was possible to determine the number of previous ovipositions in certain females by counting the number of dilatations formed from the degenerated eggs.

relict eggs were classified as parous and their ovaries were also dissected for the determination of dilatations.

Changes in the Reproductive Status and Age Structure of Wild Caught Sand Flies

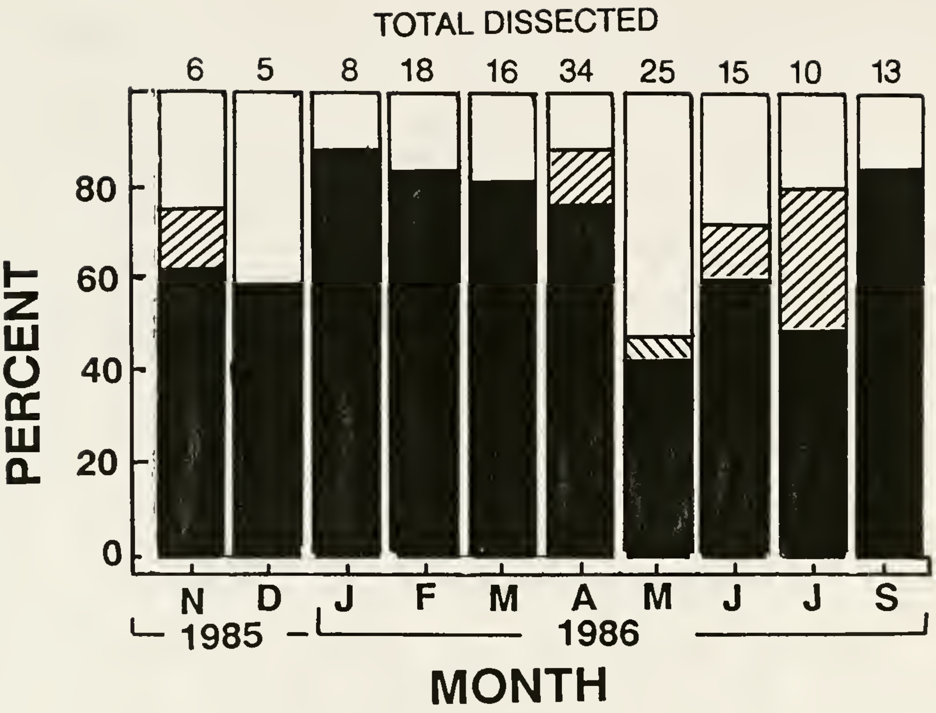
A total of 22 different species were collected from Arboledas and Durania during the present study (Table 6-2). The reproductive status (blood feeding classes) and age structure of the following sand flies: Lu. cayennensis, Lu. ovallasi, Lu. trinidadensis, Lu. shannoni, Lu. serrana, Lu. venezulensis , Lu. gomezi, and Lu. erwindonaldoi is described and their relation to Leishmania transmission is discussed in the present study.

Lutzomyia cayennensis. A large number of unfed nulliparous females with first stage ovarioles rested on the tree trunks (Figs. 6-7 & 6-8)(Table 6-3). Few fresh fed females were observed during January 1987, May, November, and January 1988.

Parous females were collected during most of the year but maximum proportion of parous females were collected during January 1988 (Table 6-3). A female that had completed 4 gonotrophic cycles was collected in August (Table 6-3). Although parity was determined in only 2 females during November, the results were presented here to show that females were gonoactive. The maximum number of females that oviposited the night before collection rested

Figure 6-9. Temporal changes in the reproductive status of *Lu. ovallesi* collected resting on tree trunks in Arboledas, Colombia during May 1985 to December 1986. ■ Unfed; ▧ fresh fed; □ late fed.

Figure 6-10. Temporal changes in the ovarian development stages of *Lu. ovallesi* collected resting on tree trunks in Arboledas, Colombia during May 1985 to December 1986. ■ First stage; ▨ second stage; ⁙ third stage; ▩ fourth stage and □ fifth stage of ovarian follicles.


TOTAL DISSECTED
6
5
8
18
16
34
25
15
10
13
PERCENT
0
20
40
60
80
N
D
J
F
M
A
M
J
J
S
1985
1986
MONTH

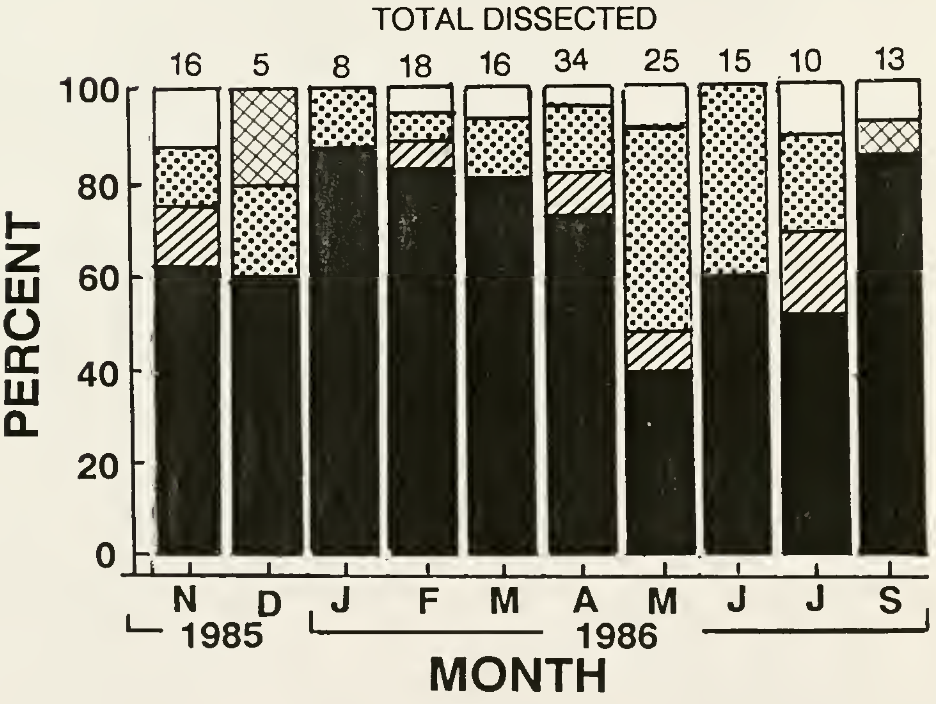
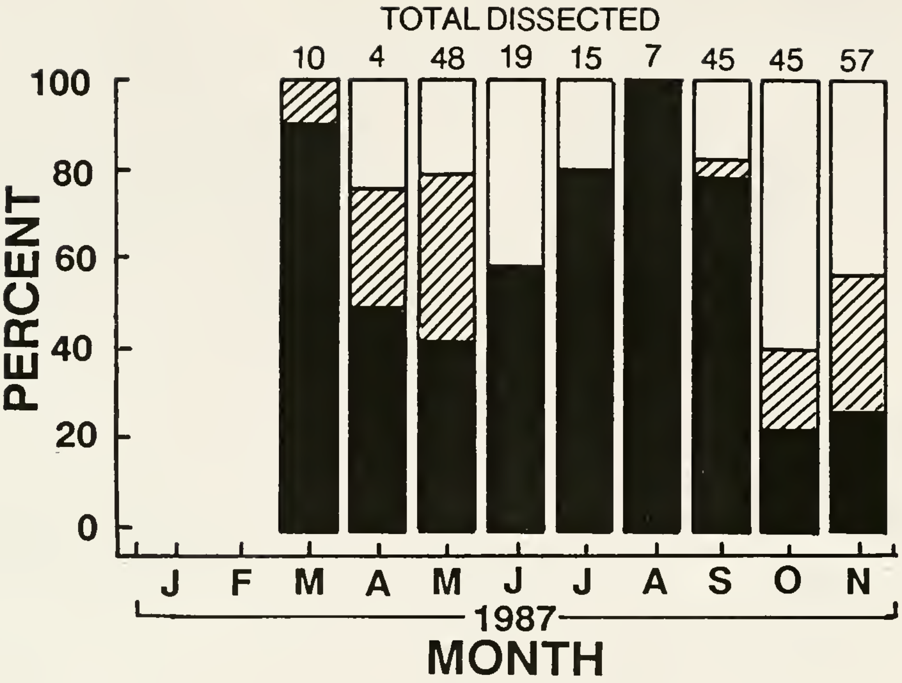

TOTAL DISSECTED
16
5
8
18
16
34
25
15
10
13
PERCENT
0
20
40
60
80
100
N
D
J
F
M
A
M
J
J
S
1985
1986
MONTH

Figure 6-11. Temporal changes in the reproductive status of *Lu. ovallesi* collected resting on tree trunks in Durania, Colombia during January 1987 to January 1988. ■ Unfed; ▨ fresh fed; ☐ late fed.

Figure 6-12. Temporal changes in the ovarian development stages of *Lu. ovallesi* collected resting on tree trunks in Durania, Colombia during January 1987 to January 1988. ■ First stage; ▨ second stage; ⬚ third stage; ▩ fourth stage and ☐ fifth stage of ovarian follicles.


TOTAL DISSECTED
10
4
48
19
15
7
45
45
57
PERCENT
100
80
60
40
20
0
J
F
M
A
M
J
J
A
S
O
N
1987
MONTH

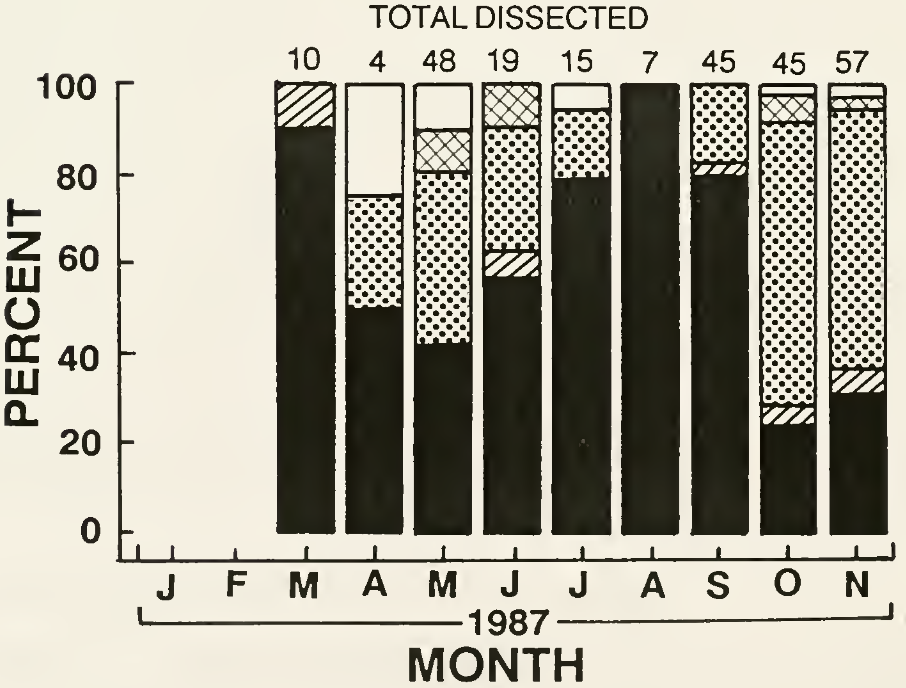

TOTAL DISSECTED
10
4
48
19
15
7
45
45
57
PERCENT
100
80
60
40
20
0
J
F
M
A
M
J
J
A
S
O
N
1987
MONTH

Table 6-4. Changes in the age structure of Lu. ovallesi collected resting on tree trunks in Arboledas, Colombia during August 1985 to September 1986.

Month	Total* Diss.	P**	Number of Dilatations				NE***	Sack**** Stage(%)
			0(%)	1(%)	2(%)	3(%)		
November	16	1(.07)	13(93)	1(7)			2	
December	5	0(.00)	4(100)				1	
January	8	0(.00)	8(100)					
February	18	1(.06)	16(94)		1(6)			
March	16	0(.00)	15(100)				1	
April	34	2(.06)	29(94)	1(3)	1(3)			
May	25	1(.05)	21(95)	1(5)			4	1(4)
June	15	1(.07)	14(93)			1(7)		
July	10	0(.00)	4(100)					
August	No dissections							
September	13	2(.15)	11(85)		1(8)	1(8)		

*Total females dissected each month. ** Parous females (proportion parous i.e, parous/nulliparous+parous). *** Not estimated; it was not possible to find any dilatations. **** Females that have recently oviposited. It was possible to determine the number of previous ovipositions in certain females by counting the number of dilatations formed from the degenerated eggs.

Table 6-5. Changes in the age structure of Lu. ovallesi, collected by different methods in Durania, Colombia during January 1987 to January 1988.

Month	Total* Diss.	P**	Number of Dilatations				NE***	Sack**** Stage(%)
			0(%)	1(%)	2(%)	3(%)		
				Tree Trunk				
March	10	1(.11)	8(89)		1(11)		1	
April	4	4(.00)	4(100)					
May	48	4(.10)	37(90)	2(5)			7	2(5)
June	19	3(.17)	15(83)	1(6)			1	2(11)
July	15	1(.07)	13(93)				1	1(6)
August	7	2(.28)	5(71)	1(14)	1(14)			
September	47	8(.17)	39(83)	2(4)	4(9)		2	4(9)
October	45	3(.08)	34(92)	3(8)			8	
November	57	3(.09)	32(91)	2(6)			22	1(3)
December	No dissections							
January	1	0	1					
				Human Bait				
April	2	0(.00)	2(100)					
May	5	0(.00)	4(100)				1	
August	23	0(.00)	23(100)					
September	7	1(.14)	6(86)					1(14)
November	15	2(.13)	13(87)	1(7)				1(7)
January	6	2(.33)	4(67)	2(33)				2(33)
				Light Trap				
July	35	4(.17)	20(83)				11	3(13)
August	15	1(.07)	13(93)		1(7)		1	
September	6	1(.17)	5(83)					1(17)

Table 6-5--extended.

*Number of total females that were dissected each month. ** Parous females (proportion parous i.e, parous/nulliparous+parous). *** Not estimated; it was not possible to find any dilatations. **** Females that have recently oviposited. It was possible to determine the number of previous ovipositions in certain females by counting the number of dilatations formed from the degenerated eggs.

on tree trunks in June and July (Table 6-3).

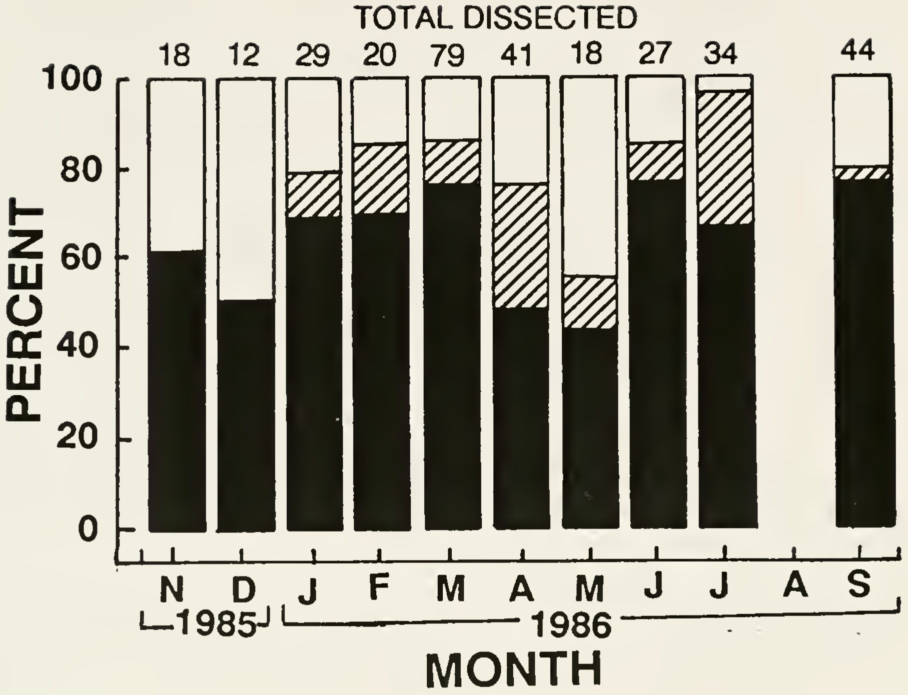
A few unfed and late fed females were collected resting in the houses. One female collected during May had ovaries developed to the third stage and another female had completed 2 gonotrophic cycles (Table 6-3). There was no difference in the parity of females that rested on tree trunks or in leaf litter during August (Table 6-3). Nulliparous females were attracted to light trap (Table 6-3). Females that had completed 2 or more gonotrophic cycles were found during March, May-August, and December.

Lutzomyia ovallesi. Tree trunks were the preferred resting sites of unfed, fresh fed, and late blood fed females at Arboledas and Durania (Figs. 6-9, 6-10, 6-11 & 6-12)(Tables 6-4 & 6-5). The maximum proportion of parous females that rested on the tree trunks at Durania were collected in August but no parous female came to bite at dusk (Table 6-5). These results suggest that parous females were biting at some other time during the night. Females were gonoactive in August because one late fed female was collected at Arboledas in this month (result not shown).

Females that had completed 2 gonotrophic cycles were resting on tree trunks in February, April, and September at Arboledas (Table 6-4). Similarly, females that had completed 2 gonotrophic cycles were collected while resting on tree trunks during March, August, and September at Durania (Table 6-5). One female with 2 dilatations was

Figure 6-13. Temporal changes in the reproductive status of *Lu*. *serrana* collected resting on tree trunks in Arboledas, Colombia during November 1985 to September 1986. ■ Unfed; ▧ fresh fed; □ late fed.

Figure 6-14. Temporal changes in the ovarian development stages of *Lu*. *serrana* collected resting on tree trunks in Arboledas, Colombia during November 1985 to September 1986. ■ First stage; ▨ second stage; ⊡ third stage; ▩ fourth stage and □ fifth stage of ovarian follicles.


TOTAL DISSECTED
18
12
29
20
79
41
18
27
34
44
100
80
60
40
20
0
PERCENT
N
D
J
F
M
A
M
J
J
A
S
1985
1986
MONTH

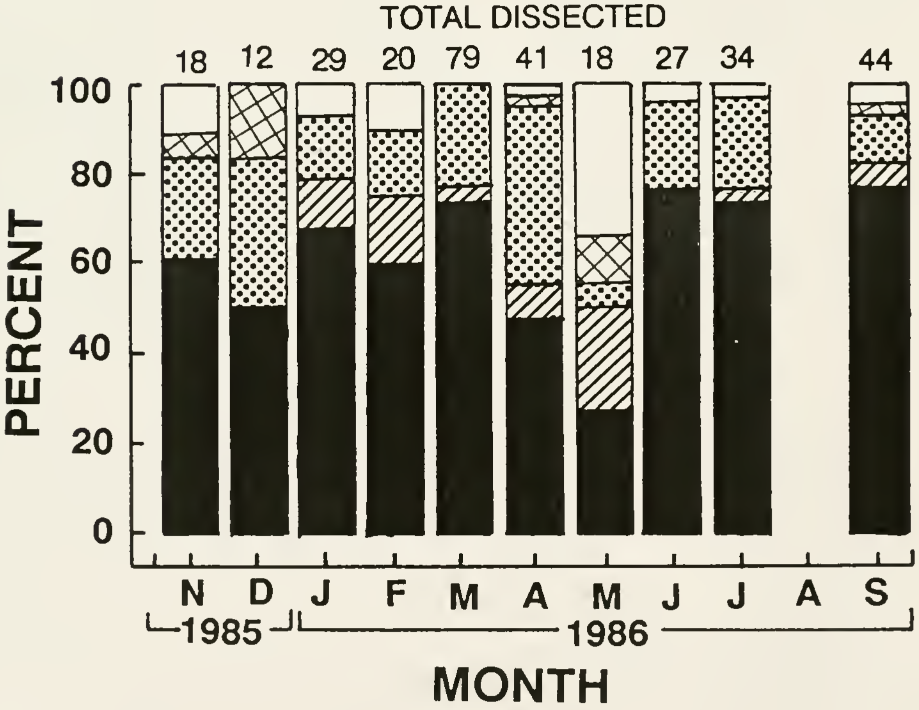

TOTAL DISSECTED
18
12
29
20
79
41
18
27
34
44
100
80
60
40
20
0
PERCENT
N
D
J
F
M
A
M
J
J
A
S
1985
1986
MONTH

Table 6-6. Age structure of *Lu*. *serrana* collected resting on tree trunks in Arboledas, Colombia during August 1985 to September 1986.

Month	Total* Diss.	P**	Number of Dilatations 0(%)	1(%)	2(%)	3(%)	NE***	Sack**** Stage(%)
			Tree Trunk					
August	5	0(.00)	5(100)					
October	2	0(.00)	2(100)					
November	18	2(.13)	14(78)	2(13)			2	
December	12	0(.00)	9(100)				3	
January	28	2(.08)	24(92)			1(4)	2	1(4)
February	20	0(.00)	17(100)				3	
March	79	3(.04)	75(95)	1(1)	1(1)		1	2(3)
April	40	5(.13)	34(87)	1(3)			2	4(11)
May	18	2(.17)	10(83)	2(2)			6	
June	27	3(.12)	23(88)				1	3(12)
July	34	2(.06)	31(94)				1	2(6)
August	No dissections							
September	44	0(.00)	43(100)				1	
			Human Bait					
August	3	0(.00)	3(100)					
June	6	0(.00)	6(100)					
July	4	0(.00)	4(100)					
August	6	0(.00)	6(100)					
September	16	3(.21)	11(79)	1(7)	1(7)		2	3(21)

Table 6-6--extended.

*Total females dissected each month. ** Parous females (proportion parous i.e, parous/nulliparous+parous). *** Not estimated; it was not possible to find any dilatations. **** Females that have recently oviposited. It was possible to determine the number of previous ovipositions in certain females by counting the number of dilatations formed from the degenerated eggs.

collected biting a volunteer during July (result not shown). Maximum number of females with sack stage were resting on tree trunk during September (Table 6-5). Females laid 3 batches of eggs during June and September at Arboledas (Table 6-4). The chances of Leishmania transmission were inhanced in September at Arboledas because 15 % of the females had completed 2-3 gonotrophic cycles (Table 6-4).

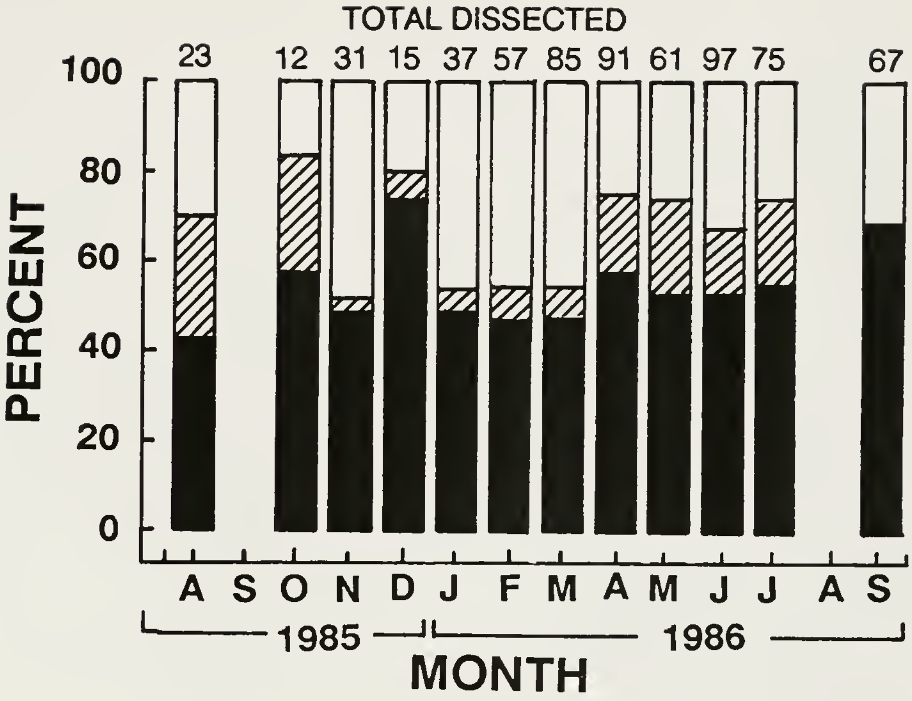
Gonotrophic discordance was observed in Lu. ovallesi during May and January at Durania. Two females out of 5 collected in May during human bait collections were late fed. Similarly, 2 females out of 6 collected during January were late fed.

Late fed females (37%), with eggs in fifth stage were attracted to light traps at Durania in July. No late fed females were collected in August and 1 female with eggs developed to 3rd stage was collected in a light trap during September.

Lutzomyia serrana. Temporal changes in reproductive status and age structure were determined only at Arboledas because the population of Lu. serrana at Durania was not large enough throughout the year except in September. The lowest number of newly emerged, unfed and nulliparous females was present during April and May 1986, a period of peak rainfall at Arboledas (Alexander 1988)(Figs. 6-2, 6-13 & 6-14). The proportion of parous females increased in the rainy season; the maximum proportion of parous females was

Figure 6-15. Temporal changes in the reproductive status of *Lu*. *shannoni* collected resting on tree trunks in Arboledas, Colombia during August 1985 to September 1986. ■ Unfed; ▨ fresh fed; □ late fed.

Figure 6-16. Temporal changes in the ovarian development stages of *Lu*. *shannoni* collected resting on tree trunks in Arboledas, Colombia during August 1985 to September 1986. ■ First stage; ▨ second stage; ⊡ third stage; ▩ fourth stage and □ fifth stage of ovarian follicles.


TOTAL DISSECTED
23
12
31
15
37
57
85
91
61
97
75
67
100
80
60
40
20
0
PERCENT
A
S
O
N
D
J
F
M
A
M
J
J
A
S
1985
1986
MONTH

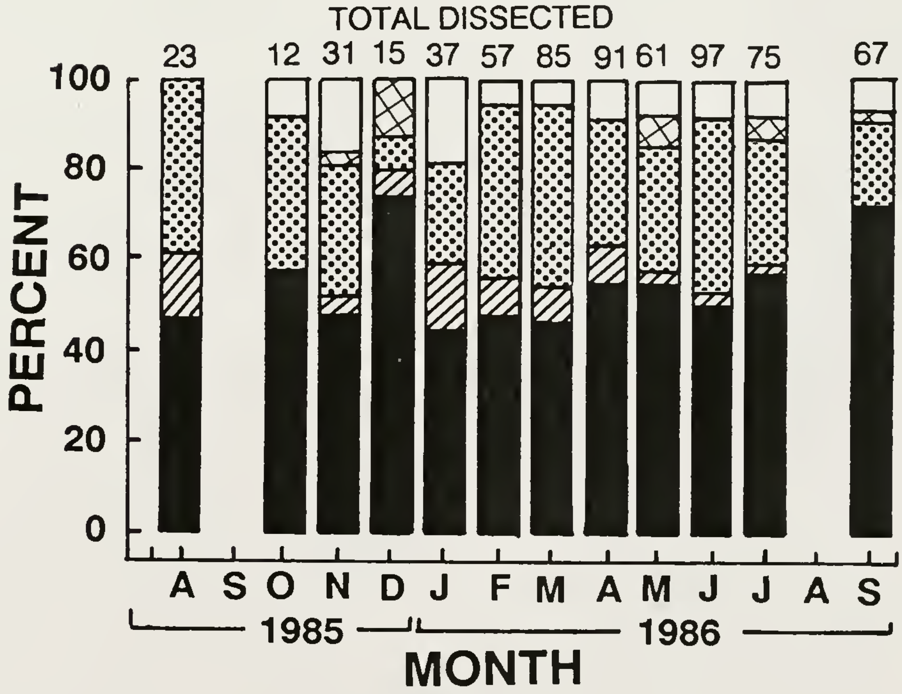
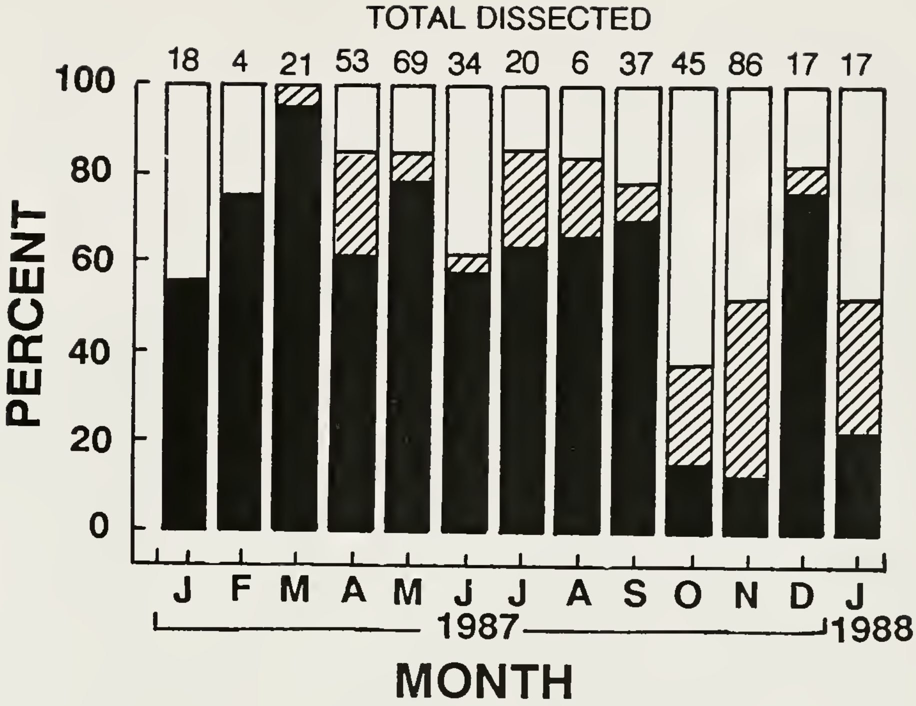

TOTAL DISSECTED
23
12
31
15
37
57
85
91
61
97
75
67
100
80
60
40
20
0
PERCENT
A
S
O
N
D
J
F
M
A
M
J
J
A
S
1985
1986
MONTH

Figure 6-17. Temporal changes in the reproductive status of *Lu*. *shannoni* collected resting on tree trunks in Durania, Colombia during January 1987 to January 1988. ■ Unfed; ▨ fresh fed and □ late fed.

Figure 6-18. Temporal changes in the ovarian development stages of *Lu*. *shannoni* collected resting on tree trunks in Durania, Colombia during January 1987 to January 1988. ■ First stage; ▨ second stage; ⊡ third stage; ▩ fourth stage and □ fifth stage of ovarian follicles.


TOTAL DISSECTED
18
4
21
53
69
34
20
6
37
45
86
17
17
100
80
60
40
20
0
PERCENT
J
F
M
A
M
J
J
A
S
O
N
D
J
1987
1988
MONTH

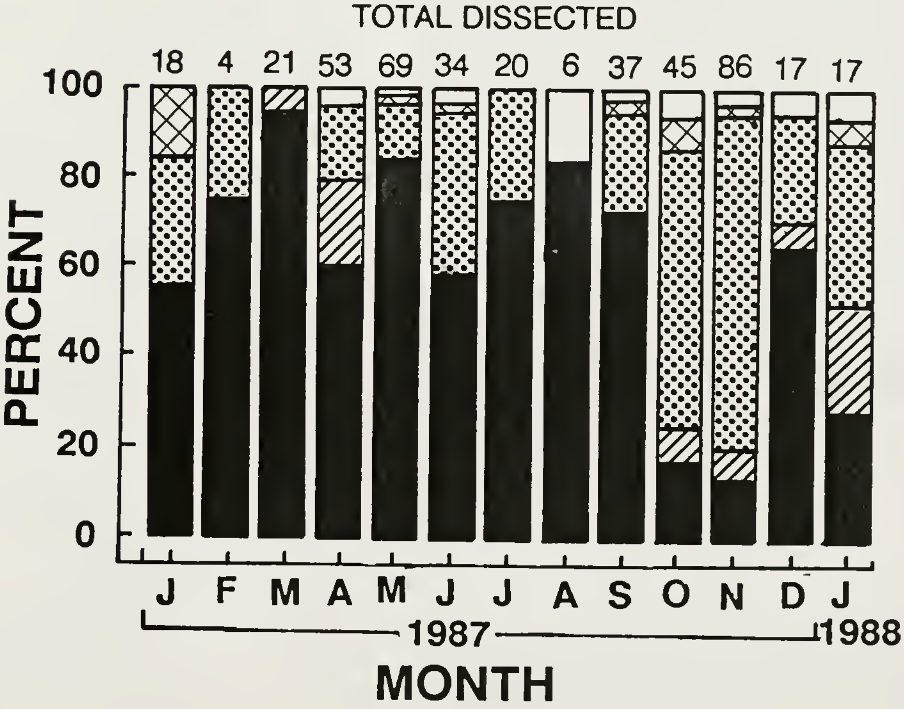

TOTAL DISSECTED
18
4
21
53
69
34
20
6
37
45
86
17
17
100
80
60
40
20
0
PERCENT
J
F
M
A
M
J
J
A
S
O
N
D
J
1987
1988
MONTH

Table 6-7. Changes in the age structure of Lu. shannoni collected resting on tree trunks and during human bait biting collections in Arboledas, Colombia during August 1985 to September 1986.

Month	Total* Diss.	P**	Number of Dilatations				NE***	Sack**** Stage(%)
			0(%)	1(%)	2(%)	3(%)		
			Tree Trunk					
August	23	3(.16)	18(86)		1(5)		2	3(14)
September	No dissections							
October	12	1(.10)	9(90)		1(10)		2	
November	31	2(.08)	24(92)	1(4)	1(4)		5	
December	15	0(.00)	15(100)					
January	37	3(.10)	27(90)				7	3(10)
February	57	8(.14)	46(85)	5(9)		1(2)	3	2(4)
March	85	11(.14)	69(86)	2(3)	2(3)		5	7(9)
April	90	10(.12)	74(88)	4(5)		1(1)	5	6(7)
May	61	9(.16)	47(84)	5(9)	2(4)		5	3(5)
June	97	13(.14)	73(78)	3(3)	4(4)		11	7(8)
July	75	8(.12)	60(88)	5(7)			6	5(7)
August	No dissections							
September	67	2(.03)	58(97)	1(2)			7	1(2)
			Human Bait					
November	4	2(.50)	2(50)	2(50)				2(50)
June	5	2(.40)	3(60)	1(20)				2(40)
July	3	1(.33)	2(67)		1(33)			1(33)
August	2	0(.00)	2(100)					
September	3	0(.00)	3(100)					

Table 6-7--extended.

*Total females dissected each month. ** Parous females (proportion parous i.e, parous/nulliparous+parous). *** Not estimated; it was not possible to find any dilatations. **** Females that have recently oviposited. It was possible to determine the number of previous ovipositions in certain females by counting the number of dilatations formed from the degenerated eggs.

Table 6-8. Changes in age structure of Lu. shannoni collected by different methods in Durania, Colombia during January 1987 to January 1988.

Month	Total* Diss.	P**	Number of Dilatations				Sack**** Stage(%)
			0(%)	1(%)	2(%)	NE***	
			Tree Trunk				
January	18	2(.11)	16(89)	1(6)	1(6)		
February	4	0(.00)	4(100)				
March	21	2(.10)	19(90)	1(5)			1(5)
April	53	2(.04)	49(96)			2	2(4)
May	69	9(.14)	56(86)	4(6)	2(3)	4	3(5)
June	34	4(.12)	29(88)	1(3)	1(3)	1	2(6)
July	20	1(.06)	16(94)			3	1(6)
August	6	0(.00)	6(100)				
September	37	5(.15)	28(85)	3(9)	2(6)	4	
October	45	1(.02)	42(98)	1(2)		2	
November	86	4(.07)	54(93)	3(5)	1(2)	28	1(2)
December	17	0(.00)	12(100)			5	
January	17	2(.12)	14(88)	1(6)	1(6)	1	
			Human Bait				
March	2	1(.50)	1(50)				1(50)
April	4	0(.00)	4(100)				
May	5	1(.20)	4(80)				1(20)
August	4	2(.50)	2(50)	1(25)			1(25)
September	9	4(.44)	5(56)	2(22)			2(22)
October	2	1(.50)	1(50)				

Table 6-8--extended.

*Total females dissected each month. ** Parous females (proportion parous i.e, parous/nulliparous+parous). *** Not estimated; it was not possible to find any dilatations. **** Females that have recently oviposited. It was possible to determine the number of previous ovipositions in certain females by counting the number of dilatations formed from the degenerated eggs.

present in May (Table 6-6). The maximum number of females that oviposited within the previous 12 hrs was collected from tree trunks in June (Table 6-6). One female (4%) completed 3 gonotrophic cycles in January. Only nulliparous females came to suck blood at dusk in August, June and July at Arboledas.

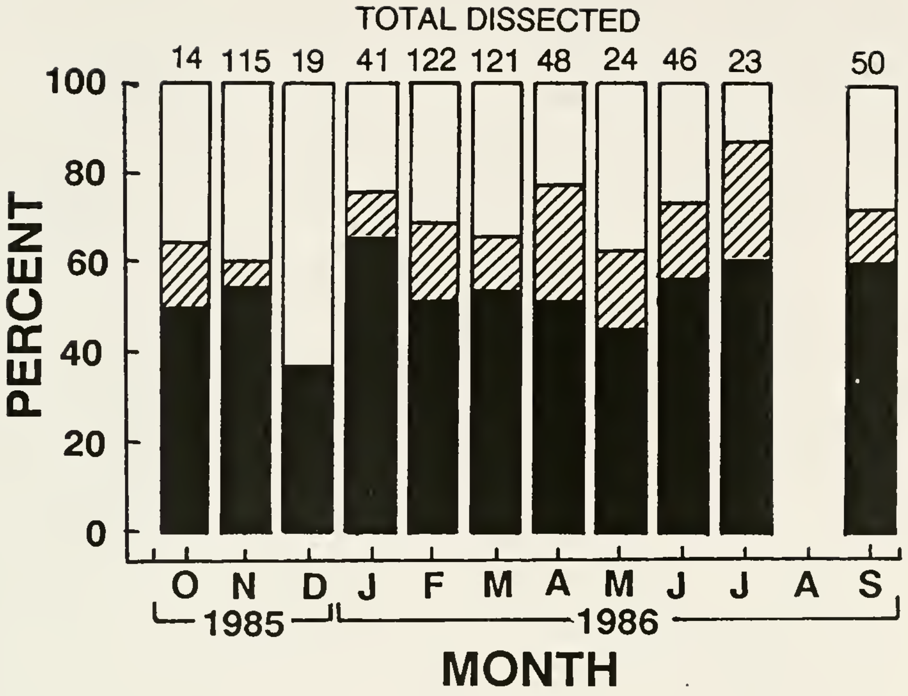
No parous females were found resting on tree during September but 3 (21%) parous females came to suck blood from the volunteers. One female had completed 2 gonotrophic cycles (Table 6-6). Similarly, 3 (21%) parous females were collected resting on tree trunks in Durania. One female completed 2 gonotrophic cycles in September.

Lutzomyia shannoni. Females were gonoactive throughout the year in Arboledas and Durania (Figs. 6-15, 6-16, 6-17 & 6-18)(Tables 6-7 & 6-8). More than 40% unfed females that had resting stage ovaries were taken resting on tree trunks. No gravid females were found in August (Figs. 6-15 & 6-16). No fresh fed females rested on tree trunks during September 1986 (Fig. 6-15).

The maximum proportion of parous females were found resting on tree trunks at Arboledas in May 1986; 16% females completed 1-2 gonotrophic cycles (Table 6-7). Females completed 2 gonotrophic cycles in August, October, and November of 1985 and March, May, and June of 1986. Females laid 3 egg batches in February and April at Arboledas (Table 6-7).

Figure 6-19. Temporal changes in the reproductive status of *Lu*. *spinicrassa* collected resting on tree trunks in Arboledas, Colombia during October 1985 to September 1986. ■ Unfed; ▨ fresh fed and □ late fed.

Figure 6-20. Temporal changes in the ovarian development stages of *Lu*. *spinicrassa* collected resting on tree trunks in Arboledas, Colombia during October 1985 to September 1986. ■ First stage; ▨ second stage; ⚃ third stage; ▩ fourth stage and □ fifth stage of ovarian follicles.


TOTAL DISSECTED
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PERCENT
0 20 40 60 80 100
O N D J F M A M J J A S
1985
1986
MONTH

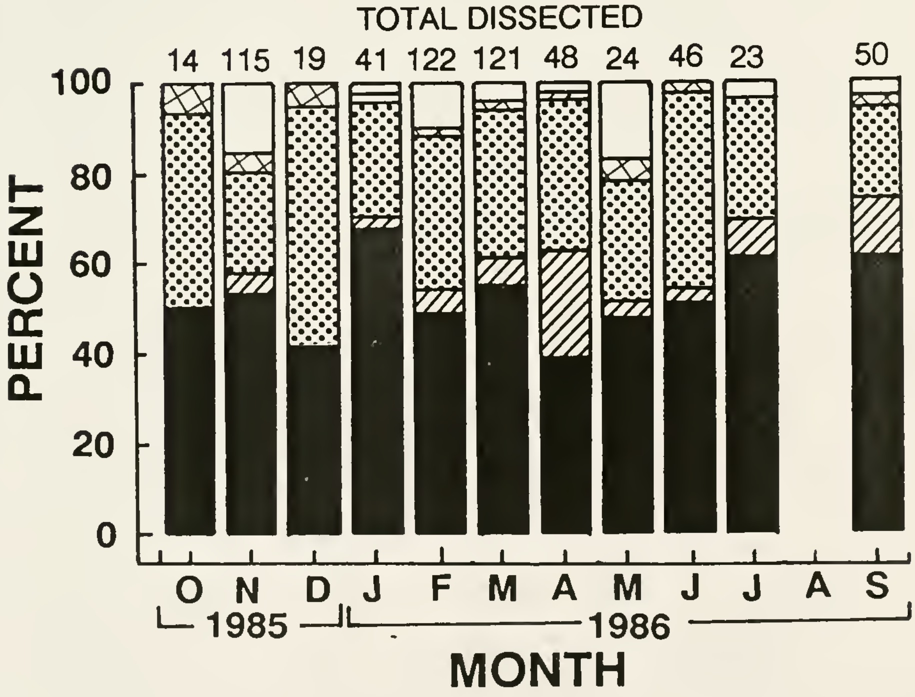
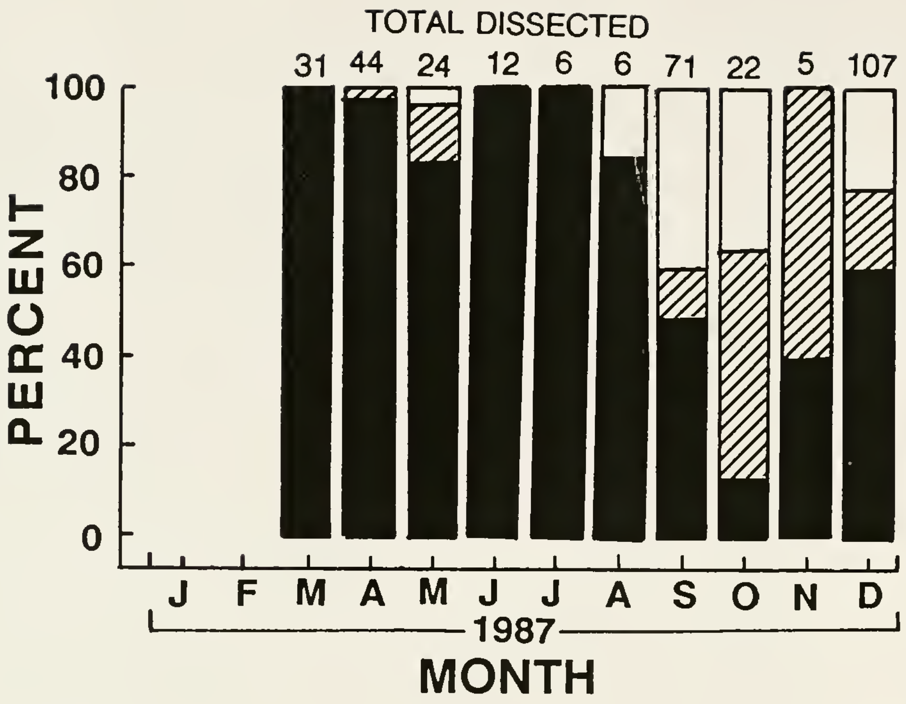
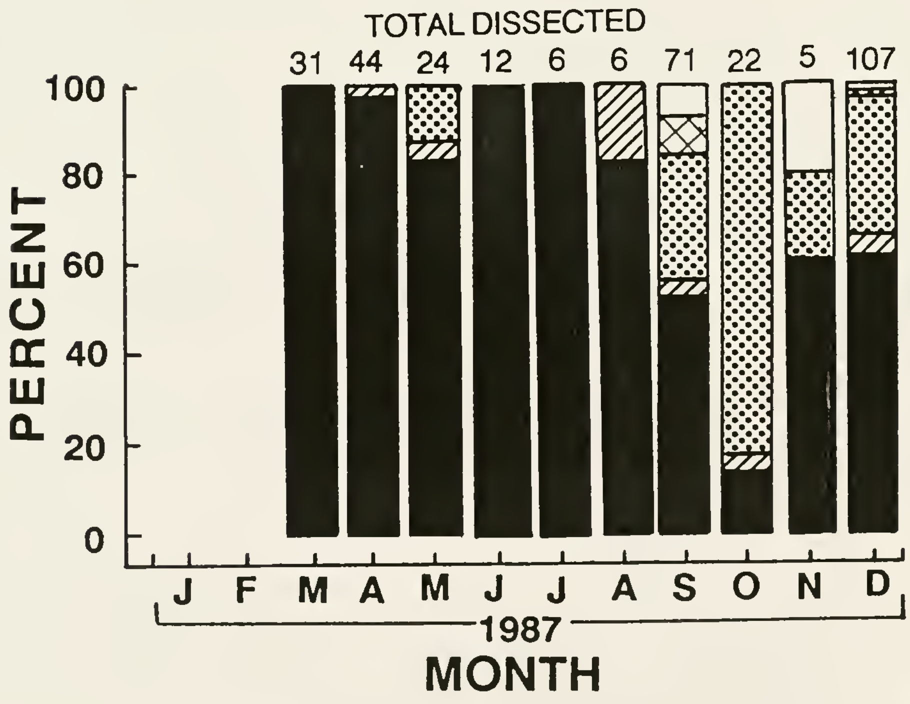

TOTAL DISSECTED
14 115 19 41 122 121 48 24 46 23 50
PERCENT
0 20 40 60 80 100
O N D J F M A M J J A S
1985
1986
MONTH

Figure 6-21. Temporal changes in the reproductive status of *Lu*. *spinicrassa* collected resting on tree trunks in Durania, Colombia during January 1987 to December 1987. ■ Unfed; ▨ fresh fed and □ late fed.

Figure 6-22. Temporal changes in the ovarian development stages of *Lu*. *spinicrassa* collected resting on tree trunks in Durania, Colombia during January 1987 to December 1987. ■ First stage; ▨ second stage; ⊡ third stage; ▩ fourth stage and □ fifth stage of ovarian follicles.


TOTAL DISSECTED
31
44
24
12
6
6
71
22
5
107
PERCENT
100
80
60
40
20
0
J
F
M
A
M
J
J
A
S
O
N
D
1987
MONTH


TOTAL DISSECTED
31
44
24
12
6
6
71
22
5
107
PERCENT
100
80
60
40
20
0
J
F
M
A
M
J
J
A
S
O
N
D
1987
MONTH

Gonoactivity pattern of females from Durania was similar to those at Arboledas in tree trunk resting females from January 1987 to January 1988. No fresh fed females were recorded resting on tree trunks in January and February 1987 and none had 5th stage eggs from January to March 1987 (Figs. 6-17 & 6-18). Females that had fifth stage eggs rested on tree trunks during August 1987. The lowest number of unfed females with first stage ovaries was found in October and November 1987. The maximum percent of females had 3rd stage ovarian follicles during these months; were nulliparous. The maximum proportion (0.15) of parous females were caught resting on tree trunks in September at Durania (Table 6-8). Females that had completed 2 gonotrophic cycles were found throughout the year. Because _Lu_. _shannoni_ is autogenous and can lay one egg batch without blood feeding, 2 parous females can be considered as potential vectors of leishmaniasis.

Parous females bit at dusk during all months except in April 1987 in Durania (Tables 6-7 & 6-8). Females that had completed two gonotrophic cycles came to bite at Arboledas in July._Lutzomyia spinicrassa_. Gonoactive females were present throughout the year at Arboledas and Durania (Figs. 6-19, 6-20, 6-21 & 6-22). A large number of females found resting on tree trunks were late fed and gravid during October-December and May at Arboledas (Figs. 6-19 & 6-20).

Table 6-9. Changes in the longevity of _Lu_. _spinicrassa_ collected resting on tree trunks and during human bait collections in Arboledas, Colombia during August 1985 to September 1986.

Month	Total* Diss.	P**	Number of Dilatations 0(%)	1(%)	2(%)	3(%)	NE***	Sack**** Stage(%)
			Tree Trunk					
August	8	1(.14)	6(86)				1	1(14)
September	No dissections							
October	14	0(.00)	13(100)				1	
November	115	12(.13)	77(87)	3(3)			26	9(10)
December	19	1(.07)	14(93)				4	1(7)
January	41	6(.15)	33(85)				2	6(15)
February	121	13(.12)	95(88)	6(6)			13	7(6)
March	121	8(.07)	102(93)	4(4)			11	4(4)
April	48	5(.11)	39(89)	2(4)			4	3(7)
May	24	1(.05)	20(95)		1(5)		3	
June	46	6(.15)	35(85)	3(7)			5	3(7)
July	23	2(.11)	17(89)	2(11)			4	
August	No Dissections							
September	50	1(.02)	46(98)	1(2)			3	
			Human Bait					
August	26	4(.15)	22(85)					4(15)
November	24	2(.08)	22(92)	2(8)	1(4)			
January	8	0(.00)	8(100)					
February	4	0(.00)	4(100)					
May	7	0(.00)	7(100)					
June	93	19(.26)	74(80)	7(8)	1(1)			17(18)
July	72	7(.10)	65(90)	3(4)	1(1)			7(10)

Table 6-9--extended.

*Total females dissected each month. ** Parous females (proportion parous i.e, parous/nulliparous+parous). *** Not estimated; it was not possible to find any dilatations. **** Females that have recently oviposited. It was possible to determine the number of previous ovipositions in certain females by counting the number of dilatations formed from the degenerated eggs.

Table 6-10. Changes in age structure of _Lu_. _spinicrassa_ collected by different methods in Durania, Colombia during January 1987 to January 1988.

Month	Total* Diss.	P**	Number of Dilatations 0(%)	1(%)	2(%)	NE***	Sack**** Stage(%)
			Tree Trunk				
January	2	0(.00)	2(100)				
March	31	6(.19)	25(81)	1(3)			5(16)
April	44	4(.09)	40(91)				4(10)
May	24	1(.04)	23(96)				1(4)
June	12	0(.00)	10(83)			2	
July	4	1(.50)	2(50)	1(25)			1(25)
August	6	1(.17)	5(83)				1(17)
September	71	7(.11)	54(89)		1(2)		6(10)
October	22	1(.05)	20(95)		1(5)		
November	5	2(.40)	3(60)	1(20)			
December	77	5(.06)	72(82)	3(3)	2(2)		11(13)
			Human Bait				
January	2	1(.50)	1(50)	1(50)			
March	17	1(.06)	16(94)	1(6)			1(6)
April	8	2(.25)	6(75)	1(2)	1(2)		
May	3	0(.00)	3(100)				
June	3	1(.33)	2(67)				1(33)
August	224	31(.15)	171(85)	5(3)	2(1)		25(11)
September	353	66(.19)	287(81)	17(5)	3(1)		52(15)
October	72	9(.13)	63(87)	1(1)	1(1)		7(10)
November	58	2(.03)	55(95)	1(2)		1	1(2)
December	35	5(.14)	30(86)	1(3)			4(11)

Table 6-10--extended.

Month	Total* Diss.	P**	Number of Dilatations			NE***	Sack**** Stage(%)
			0(%)	1(%)	2(%)		
			Light Trap				
July	13	2(.15)	11(85)				2(15)
August	23	2(.10)	18(90)			3	2(10)
September	31	3(.10)	26(90)			2	1(3)
			Indoor Resting				
March	6	0(.00)	6(100)				
August	2	0(.00)	2(100)				
September	3	0(.00)	3(100)				
October	4	1(.25)	3(75)				1(25)
November	5	0(.00)	5(100)				
			Rocks				
August	10	0(.00)	10(100)				
September	32	3(.09)	29(91)	2(6)			1(3)

*Total females dissected each month. ** Parous females (proportion parous i.e, parous/nulliparous+parous). *** Not estimated; it was not possible to find any dilatations. **** Females that have recently oviposited. It was possible to determine the number of previous ovipositions in certain females by counting the number of dilatations formed from the degenerated eggs.

Females of all trophic status used tree trunks as their diurnal resting site (Figs. 6-19 & 6-20).

The maximum proportion of parous females were found on tree trunks in January and June 1986 at Arboledas. During January, a maximum (15%) of females oviposited a night before collection (Table 6-9). The minimum proportion of parous females was collected in September (Table 6-9). One female (5%) completed 2 gonotrophic cycles in May.

Parous females attacked human volunteers during August, November, June, and July. In June, 18% females refed after oviposition and 20% of the biting females were parous. Four percent of females that came to bite during November had completed 2 gonotrophic cycles. Similarly, females that had completed two gonotrophic cycles came to refeed on volunteers during June and July (Table 6-9).

No blood fed females were taken from the tree trunks during March, June, and July at Durania, and this could be due to small sample size (Figs. 6-21 & 6-22). Parous females were collected in March and July (Table 6-10). A high proportion of parous females were collected during July and November but again the sample sizes were to small to draw a definite conclusions. Females that had completed 2 gonotrophic cycles were collected in September, October, and December and the greatest number (5%) of such females were collected during October. A large proportion of females that had oviposited recently, used tree trunks as their resting sites during March-May, July-August, and December (Table 6-10).

Parous females bit people throughout the year (Table 6-10). Females that had completed 2 gonotrophic cycles took a third blood meal during April, August, September, and October (Table 6-10). A greater proportion of parous females were taken in biting collections than those collected resting on tree trunks during October (Table 6-10).

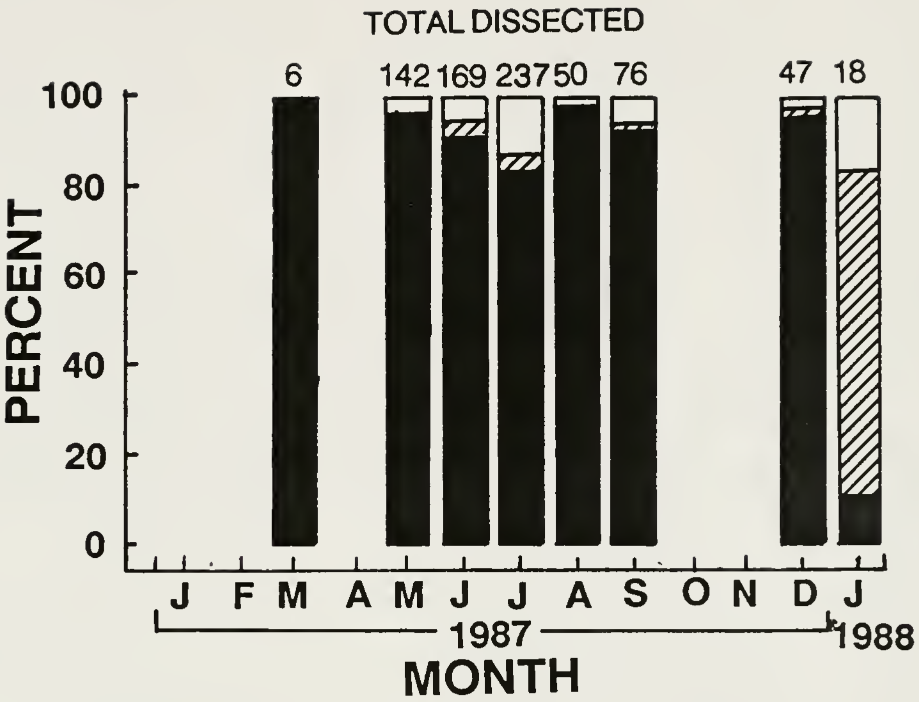
A small percentage of females that had third and fifth stage eggs were taken in biting collections. Thus, 0.9%, 6%, 1.4%, and 2.2% females that had third stage eggs in their ovaries were biting in September, October, November, and December respectively. Similarly, 1.4% and 1.7% females that came to bite had fifth stage ovarian follicles in November and December. Such females were either showing gonotrophic discordance or were simply opportunistic.

Few gonoactive females were collected in resting collections. Rocks were used as resting sites by unfed, fresh fed, and late fed females.

Lutzomyia trinidadensis. A large number of _Lu_. _trinidadensis_ was caught resting on tree trunks and dissected during May, September, December 1987, and January 1988. Results from other months are also presented but these should be viewed with caution due to small sample sizes. The maximum number of females in tree trunk resting

Figure 6-23. Temporal changes in the reproductive status of *Lu*. *trinidadensis* collected resting on tree trunks in Durania, Colombia during January 1987 to January 1988. ■ Unfed; ▨ fresh fed and □ late fed.

Figure 6-24. Temporal changes in the ovarian development stages of *Lu*. *trinidadensis* collected resting on tree trunks in Durania, Colombia during January 1987 to January 1988. ■ First stage; ▨ second stage; ⬚ third stage; ▩ fourth stage and □ fifth stage of ovarian follicles.


TOTAL DISSECTED
6
142
169
237
50
76
47
18
PERCENT
100
80
60
40
20
0
J
F
M
A
M
J
J
A
S
O
N
D
J
1987
1988
MONTH

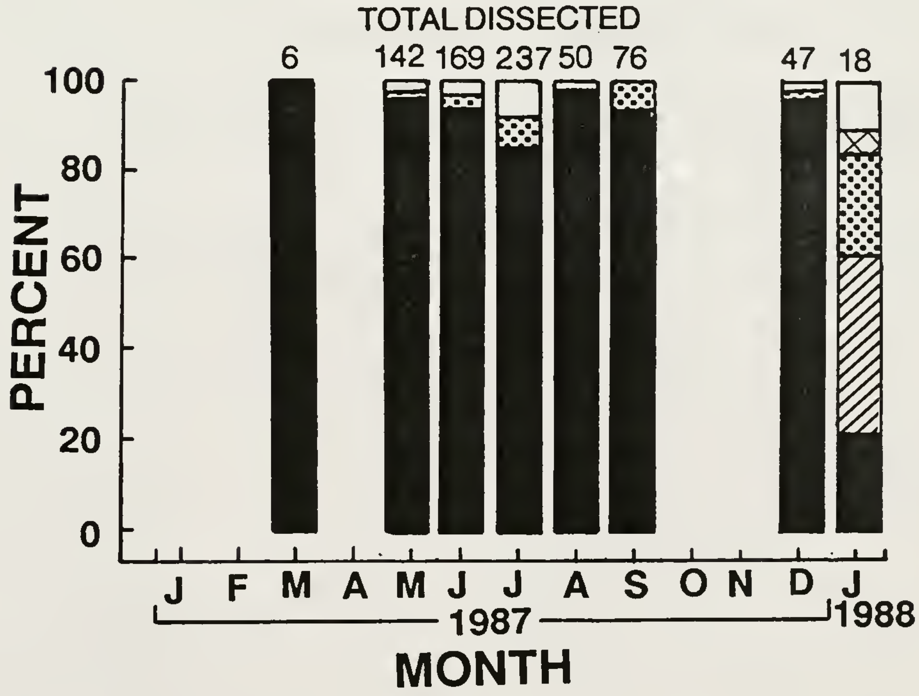

TOTAL DISSECTED
6
142
169
237
50
76
47
18
PERCENT
100
80
60
40
20
0
J
F
M
A
M
J
J
A
S
O
N
D
J
1987
1988
MONTH

Table 6-11. Age structure of *Lu. trinidadensis* collected by different methods in Durania, Colombia during January 1987 to January 1988.

Month	Total* Diss.	P**	Number of Dilatations 0(%)	1(%)	2(%)	3(%)	4(%)	NE***	Sack**** Stage(%)
			Tree Trunk Resting						
March	6	1(.17)	5(83)	1(17.0)				1	
April	2	0(.00)	2(100)						
May	142	5(.04)	128(96)	4(3.0)	1(0.7)			9	1(0.8)
June	169	9(.05)	155(95)	1(0.6)				5	8(5.0)
July	237	19(.09)	188(91)	16(8.0)	2(1.0)		1(0.4)	30	8(4.0)
August	50	4(.08)	45(92)	4(8.0)				1	1(2.0)
September	76	11(.15)	64(85)	5(7.0)	1(1.0)		1(1.0)	5	5(7.0)
November	4	0(.00)	4(100)						
December	24	1(.04)	22(96)	1(4.0)				1	
January	18	0(.00)	12(100)					6	
February	3	0(.00)	3(100)						
			Human Bait						
January	2	0(.00)	2(100)						
May	3	0(.00)	3(100)						
			Light Trap						
July	35	7(.20)	28(80)	4(11)					5(14)
September	5	1(.20)	4(80)	1(20)					

Table 6-11--extended.

Month	Total* Diss.	P**	Number of Dilatations 0(%)	1(%)	2(%)	3(%)	4(%)	NE***	Sack**** Stage(%)
			Rocks						
September	2	0(.00)	2(100)						
December	13	0(.00)	13(100)						
			Indoor Resting						
May	18	2(.12)	15(83)	2(12)				1	
June	6	0(.00)	6(100)						
July	14	1(.07)	13(93)	1(7)					
August	40	4(.10)	35(88)	2(5)				1	2(5)
September	33	2(.06)	30(91)	2(6)					1(3)

*Total females dissected each month. ** Parous females (proportion parous i.e, parous/nulliparous+parous). *** Not estimated; it was not possible to find any dilatations. **** Females that had recently oviposited. It was possible to determine the number of previous ovipositions in certain females by counting the number of dilatations formed from the degenerated eggs.

Table 6-12. Changes in the age structure of *Lu*. *venezuelensis* collected by different methods in Durania, Colombia during January 1987 to January 1988.

Month	Total* Diss.	P**	Number of Dilatations				NE***	Sack**** Stage(%)
			0(%)	1(%)	2(%)	3(%)		
			Tree Trunk Resting					
April	2	1(.50)	1(50)	1(50)				
May	19	4(.21)	15(79)			1(5)	3	
June	10	0(.00)	9(100)				1	
July	7	2(.29)	5(71)	1(14)				1(14)
August	4	0(.00)	4(100)					
September	18	1(.05)	15(94)			1(6)	2	1(6)
October	18	3(.17)	14(78)				1	3(17)
November	10	1(.10)	9(90)					1(10)
December	34	3(.17)	31(91)					3(9)
January	5	1(.10)	3(75)				1	1(25)
			Light Trap					
July	12	3(.27)	8(73)				1	3(27)
August	2	0(.00)	2(100)					
			Rocks					
December	60	3(.05)	48(80)	2(3)			7	3(2)
			Indoor Resting					
May	5	1(.25)	3(75)				1	

Table 6-12-extended.

Month	Total* Diss.	P**	Number of Dilatations					
			0(%)	1(%)	2(%)	3(%)	NE***	Sack**** Stage(%)
			Indoor Resting					
June	2	0(.00)	2(100)					
July	6	1(.17)	5(83)	1(17)				
August	2	0(.00)	2(100)					

* Total females dissected each month. ** Parous females (proportion parous i.e, parous/nulliparous+parous). *** Not estimated; it was not possible to find any dilatations. **** Females that have recently oviposited. It was possible to determine the number of previous ovipositions in certain females by counting the number of dilatations formed from the degenerated eggs.

collections were unfed, having ovarian follicle development to stage 1 (Figs. 6-23 & 6-24). A small number of fresh and late fed females rested on the tree trunks during most of the year except in January 1988 (Fig. 6-23). Maximum numbers of parous females and females that had oviposited during the previous night were collected during September (Table 6-11). Females laid two egg batches in May, July, and September and up to 4 gonotrophic cycles were completed during July and September (Table 6-11).

The number of females in biting collections was too small to draw any conclusion but parous females were attracted to light traps (Table 6-11). The proportion of such females collected in July and September was higher when compared to tree trunk resting collections. Low numbers of fresh fed females were collected in indoor resting collections.

Lutzomyia venezuelensis. Few Lu. venezulensis were dissected from January 1987 to January 1988. However, the results for certain months are presented to show that parous females were present during those months and also that the proportion of parous females does not have any meaning in certain months (Table 6-12). Females that were resting on tree trunks completed three gonotrophic cycles during May and September. A higher proportion of parous females were found resting on tree trunks compared to those resting in rocks crevices during December (Table. 6-12).

Table 6-13. Changes in the age structure of _Lu_. _gomezi_ collected during human bait collection from Arboledas during August 1985 to September 1986.

Month	Total* Diss.	P**	Number of Dilatations 0(%)	1(%)	2(%)	3(%)	NE***	Sack**** Stage(%)
August	22	1(.05)	21(95)					1(5)
November	5	0(.00)	5(100)					
March	3	0(.00)	3(100)					
April	5	0(.00)	5(100)					
May	10	2(.20)	8(80)	2(20)				
June	15	4(.27)	11(73)					4(27)
July	37	7(.19)	29(78)	1(3)	3(8)	1(3)		3(7)
August	16	1(.06)	15(94)					1(6)
September	10	0(.00)	10(100)					

*Total females dissected each month. ** Parous females (proportion parous i.e, parous/nulliparous+parous). *** Not estimated; it was not possible to find any dilatations. **** Females that have recently oviposited. It was possible to determine the number of previous ovipositions in certain females by counting the number of dilatations formed from the degenerated eggs.

Table 6-14. Changes in the age structure of Lu. erwindonaldoi collected resting on tree trunks and in human bait collection at Durania in March 1987 to October 1987.

Month	Total* Diss.	P**	Number of Dilatations 0(%)	1(%)	2(%)	3(%)	4(%)	NE***	Sack**** Stage(%)
					Tree Trunk				
March	18	4(.27)	11(73)	1(7)	1(7)			4	2(13)
June	3	2(100)	0(.00)		2(100)			1	
July	1	1	0(.00)		1				
					Human bait				
March	1	1							1
April	18	8(.44)	10(56)	2(11)	1(6)				5(28)
May	9	3(.33)	6(67)		1(11)				2(22)
August	15	5(.33)	10(66)	3(20)	1(7)		1(7)		1(7)
September	50	15(.36)	27(64)	11(26)				8	16(38)
October	3	1(.33)	2(67)						1(33)

*Total females dissected each month. ** Parous females (proportion parous i.e, parous/nulliparous+parous). *** Not estimated; it was not possible to find any dilatations. **** Females that have recently oviposited. It was possible to determine the number of previous ovipositions in certain females by counting the number of dilatations formed from the degenerated eggs.

Lutzomyia gomezi. The highest proportion of parous Lu. gomezi was collected in June during human bait collections (Table 6-13). During July, females that had completed 1-3 gonotrophic cycles were collected biting the volunteers. Small numbers of females were collected in human bait collections at Durania; in April one female (out of 8 dissected) had completed 2 gonotrophic cycles. No late fed females came to bite people during the present collection times. Similarly, uniparous females were collected biting volunteers in August and September.

Lutzomyia erwindonaldoi. Low numbers of Lu. erwindonaldoi were found resting on tree trunks at Durania. The few females dissected had completed two gonotrophic cycles in March, June, and July 1987. Parous females came to bite at dusk; 1(7%) females completed 4 gonotrophic cycles in August. Females that had oviposited twice came to blood feed for the third time in April, May, and August (Table 6-14).

Discussion

Ovarian follicular development of Lu. anthophora was similar to that of some of the mosquito species. The ovarian follicle development stages proposed by Christophers (1911) as modified by Mer (1936) were also applicable to sand flies. The dilatation technique (Detinova 1962) was used successfully to age grade 8 Lutzomyia species from

Table 6-15. Number of gonotrophic cycles completed by different _Lutzomyia_ species during 1985-1988 in Colombia.

Species	Dilatations*	Month											
		J	F	M	A	M	J	J	A	S	O	N	D
_Lu_. _cayennensis_	1	*		*	*	*	*	*	*	*		*	*
	2			*	*	*	*	*	*				*
	3						*		*				
	4								*				
_Lu_. _ovallesi_	1	*	*	*	*	*	*	*	*	*	*	*	
	2		*	*	*		*		*	*			
	3						*			*			
	4												
_Lu_. _serrana_	1	*		*	*	*	*	*		*		*	
	2	*		*						*			
	3												
	4												
_Lu_. _shannoni_	1	*	*	*	*	*	*	*	*	*	*	*	
	2	*	*	*	*	*	*	*	*	*	*	*	
	3		*		*								
	4												
_Lu_. _spinicrassa_	1	*	*	*	*	*	*	*	*	*	*	*	*
	2				*	*	*	*	*	*	*	*	*
	3												
	4												

Table 6-15--extended.

Species	Dilatations*	J	F	M	A	M	J	J	A	S	O	N	D
Lu. trinidadensis	1			*		*	*	*	*	*	ND		*
	2					*		*		*			
	3							*		*			
	4							*		*			
Lu. gomezi	1	ND	ND			*	*	*	*		ND		ND
	2							*					
	3							*					
	4												
Lu. venezuelensis	1		*	ND	*	*	*	*		*	*	*	*
	2						*			*			
	3						*			*			
	4												
Lu. erwindonlodoi	1	ND	ND	*	*	*	*	*	*	*	*	ND	ND
	2			*	*	*	*	*	*				
	3								*				
	4								*				

* Month with no astrisk contained nulliparous females. ND = no dissections.

Colombia. The presence of accessory gland fluid in the accessory glands of females was not considered a reliable method because nulliparous Lu. anthophora developed this fluid after blood feeding and the amount of fluid increased as the egg development proceeded.

Lutzomyia spinicrassa is a probable vector of L. braziliensis at Arboledas (Young et al. 1987) and the present study supports this conclusion due to the presence of potentially dangerous females during most of the year (Table 15). Females were gonoactive throughout the year at both Arboledas and Durania. A high proportion of late fed females that were gravid was present during February, May, and November when maximum rainfall occurred at Arboledas. This might have resulted in better survival of gravid females. Another, less probable possibility is that gravid females preferentially rested in animal burrows at ground level that became water logged during these months, thus, forcing flies to rest on tree trunks.

Few Lu. spinicrassa were present during January and July (Alexander 1988), but maximum proportion of parous females rested on tree trunks during this time. During April, June, July, and August, females completed 2 gonotrophic cycles and one fly was caught that harbored L. brazilensis infection (Young et al. 1987).

In Durania, flies completed two gonotrophic cycles in April, August, September, and October. Only a few flies were found resting indoors and one parous fly was found In October. Lutzomyia spinicrassa has a bimodal pattern of seasonal abundance at Arboledas, with population peaks occurring during the wettest months (February-April and October-December). Alexander (1988) suggested that immediate increases in population during the rainy season were due to emergence of adults that were quiescent in larval, pupal or the adult stage during the dry season. In my opinion, more fresh fed females survived to the gravid stage during November, February, and March or, less probably they were forced out of their preferred resting sites and forced to rest on tree trunks. Potentially dangerous populations of Lu. spinicrassa were present during April to November at Arboledas and Durania.

Lutzomyia spinicrassa took multiple blood meals during September to December. Half gravid (with 3rd stage ovariole) and gravid (with fifth stage ovarioles) females attacked volunteers during these month. Multiple blood feeding during one gonotrophic cycle was also observed for Ph. papatasi in the laboratory (Magnarelli et al. 1984). Such behavior would increase chances of Leishmania transmission by uniparous females. Females could fly up to 84 m in search of a blood meal (Alexander 1988), thus, increasing the spread of the disease after each feeding.

Females of another species, Lu. sauroida, were structurally indistinguishable from Lu. spinicrassa but few males of this species were collected resting on tree trunks at Arboledas. It is possible that females of this species were overlooked at Arboledas and were identified as *Lu. spinicrassa*.

Lutzomyia gomezi is a proven vector of cutaneous leishmaniasis in Panama. It's anthropophilic behavior is well documented in literature (Chaniotis et al. 1971; Werner & Barreto 1981; Alexander 1988), and is a suspected vector in Colombia (Porter & De Foliart 1981). It also bites man, cows and pigs in Venezuela (Feliciangeli 1987).

In Panama *Lu. gomezi*, is considered as a secondary vector of *Leishmania braziliensis panamensis* (Johnson et al. 1963). It was infected with *Leishmania braziliensis panamensis*, and different strains of *L. mexicana* from Belize, Guatemala and Panama in the laboratory (Christensen & Herrer 1980). Development of *L. mexicana* is completed within 5 days and mouthparts are infected at this time. Thus, it can transmit leishmaniasis during May-September at Colombia. But in the present study, tree resting collection ranged from 0.2 to 1 % of the total sand fly population at Durania, so it was of minor importance in this area. Seasonal bimodality in Arboledas and population peaks of this species were observed in the two rainy seasons (Alexander 1988). On the other hand, Chaniotis et al. (1971) considered it to be a dry season species in Panama with peak populations from January-February.

Lutzomyia ovallesi, an anthropophilic species that bites during the day if disturbed and enters human habitations at night to blood feed, was present at both Arboledas and Durania. Lu. ovallesi usually bites man at ground level (Williams 1970). It is attracted to light traps (Chaniotis et al. 1975; Rutledge et al. 1975; Young 1979; Alexander 1988). The attraction of certain sand flies to light in or near houses may increase Leishmania transmission (Lewis 1971). The maximum proportion parous females were found during August 1987 in Durania. Females completed three gonotrophic cycles during June and September; 2 gonotrophic cycles were completed in February-April, June, and August (Table 6-15).

Lutzomyia ovallesi has a bimodel pattern of seasonal abundance at Arboledas with highest populations recorded from October to December (Alexander 1988). Females resting on tree trunks in Durania comprised a small percent of total sand flies in May (7%), June (4%), July (4%), August (3%), and September (9%). In Durania, females laid 2 batches of eggs in March, August, September, and October. More parous females came to bite at dusk in September, November, and January compared to August. Lutzomyia ovallesi could transmit leishmaniasis in February to April, June, and August-September when it completed 2 or more gonotrophic cycles (Table 6-15). Time required for extrinsic development of different Leishmania species varies in different vectors and during different seasons. No information is available about the rate or extrinsic incubation time for the development of *Leishmania* in *Lu. ovallesi*. If uniparous females can transmit leishmaniasis than *Lu. ovallesi* can serve as a vector of leishmaniasis throughout the year.

Williams (1966) suggested that at the end of rainy season in Belize (British Houndras), *Lu. ovallesi* was active in *Leishmania mexicana* transmission to the forest workers during the early hours of day . At Arboledas and Durania, coffee is harvested to a small extent in May and June but mainly in November (Alexander 1988). Thus, it could be an important vector in June when it completes 3 gonotropohic cycles and a minor vector in November (Table 6-15). The small population of *Lu. ovallesi* throughout the year however, lessens its importance as the main vector at Arboledas or Durania.

Lutzomyia ovallesi feeds on man and the kinkajou, *Potos flavus* (Carnivora: Procyonidae) in Panama. Christensen et al. (1983) found that it feeds on sloths, anteaters and armadillos in Panama. In Venezuela, females bite men, pigs and cows but other hosts remain unknown (Feliciangeli 1987). Although it is anthropophilic throughout its range (Mexico to Trinidad), *Lu. ovallesi* is not considered one of the most important vectors of *Leishmania* in Panama (Chaniotis 1974).

Lutzomyia cayennensis, a lizard feeder, is not considered a likely vector of leishmaniasis in Colombia or elsewhere. Lutzomyia cayennensis completed up to three gonotrophic cycle in June when females comprised 39% of the total number of sand flies collected in Durania. They comprised a large percentage of all sand flies collected during May (27%), June(39%), July (22%), August (17%) and September (10%) at Durania, Colombia.

Females completed up to 4 gonotrophic cycles (7% females) in August (Table 6-15). Although Lu. cayennensis are lizard feeders, a female that had completed 2 gonotrophic cycles in August came to bite a collector at dusk. Parous females were found resting indoors, under rocks, and in leaf litter.

Most of the year no fresh fed females were found on the tree trunks but they were recorded in collections during January, May, and November. It might be possible that females rest near to their hosts, in the tree canopy after blood feeding. Newly emerged unfed females were found resting on the tree trunks throughout the year. Females that oviposited the night before their collection were found on tree trunks from April to October, thus, suggesting that they were gonoactive during these month.

Lutzomyia serrana originally described from the Amazon Basin, occurs from Mexico and Central America to Brazil. Females oviposit between the buttress roots of trees where larvae were found (Hanson 1968). The lowest percentage of unfed and nulliparous females was present during April and May 1986, a period of peak rainfall at Arboledas. This may be due to larval and pupal mortality caused by drowning in their breeding sites.

The maximum proportion of parous females were present in May, suggesting that high humidity increased their chances of survival. No parous females were collected on tree trunks in September, but they were taken biting men. This indicates that diapause does not occur in adults of this species. Potentially dangerous females (i.e, those that had completed two gonotrophic cycles) were present in January and March (Table 6-15).

Lutzomyia serrana also feeds on rodents, edentates and opossums (Tesh et al. 1971b, 1972). Populations of this species may vary genetically because Porter and Defoliart (1981) collected only 10 females in human bait collections throughout one year at Rio Anori (Antioquia), Colombia.

Lutzomyia shannoni, the most widespread American sand fly, has a bimodal pattern of seasonal abundance at Arboledas, with maxima recorded during the two rainy seasons (Alexander 1988). There, Lu. shannoni was a dominant species in May, June, and July. The second population peak occurred in February (Alexander 1988). Females are capable of flying up to 32 m. Attraction to light traps and human bait varies among different population.

In Durania _Lu_. _shannoni_ comprised a substantial portion of the total sand flies that were collected resting on tree trunks. During May (13%), June (8%), July (4%), August (3%), September (12%), October (37%), November (77%), December (3%), and January (36%) of the sand fly population was composed of _Lu_. _shannoni_. In Arboledas, the maximum percentage of parous females (16%) were found during July when the population was most abundant. Females were capable of transmission throughout the year with maximum chances during February and April (Table 6-15).

Lutzomyia _shannoni_ is an autogenous species therefore, females that had completed at least 2 gonotrophic cycles would be potentially dangerous as vectors. In human bait collections, a large proportion (0.20 to 0.50) was comprised of parous females at both Arboledas and Durania. Lewis (1965) also reported a high parous rate of _Lu_. _shannoni_ females in biting collections during the early hours of the evening compared to later hours. Williams (1966) showed that _Lu_. _shannoni_ had two peaks of nocturnal biting activity and no diurnal activity.

Lutzomyia _shannoni_ was collected in human bait collections in Costa Rica and was suspected as a vector of _Leishmania_ _mexicana_ (WHO 1984). It feeds on rodents, birds, reptiles, amphibians, tree porcupine, sloths, and mammals other than rodents (Tesh et al. 1971b; Tesh et al. 1972; Christensen et al. 1983).

Lutzomyia trinidadensis was dissected in large numbers during May to September and December at Durania. Most of the tree trunk resting flies were unfed and 80-100 % were nulliparous throughout the year. Small numbers of fresh fed females rested on tree trunks, thus, indicating that most of the blood fed portion of the population was resting else where. Females completed four gonotrophic cycles in July and September. Females comprised a large percentage of all sand flies collected in May (43%), June (40%), July (64%), August (66%), September (28%), October (.85), November(8%), December (11%), and January 1988 (3%).

In Belize, females of Lu. trinidadensis preferentially rested in the upper foliage of trees where most of their reptilian hosts rest (Williams 1970). He also showed that most of the males remain at ground level. It might be possible that during the present study fresh fed females were also resting in the canopy of trees. Earlier studies in Panama showed that Lu. trinidadensis feeds primarily on reptiles (Johnson & Hertig 1961; McConnell & Correa 1964). Unfed, fresh, and late fed nulliparous females were found resting on tree trunks in January.

Parous and nulliparous females were present inside houses but their biting behavior there is not known. In northern Venezuela, Lu. trinidadensis was found biting man and pigs (Feliciangeli 1987). Potentially dangerous populations of Lu. trinidadensis at Durania were found during March, May to September, and December (Table 6-15).

Low numbers of Lu. venezuelensis were present at Durania throughout the year. Females completed three gonotrophic cycles in May and September 1987. This species is suspected to feed on reptiles (Williams 1970).

Lutzomyia erwindonaldoi completed 2-3 gonotrophic cycles during March-August 1987. A female that had oviposited 4 times was attracted to a volunteer in August. Due to its long survival and anthropophilic behavior it could serve as a vector of leishmaniasis in Colombia.

In the above discussion, females that completed 2 gonotrophic cycles were considered potentially dangerous as leishmaniasis vectors. Perhaps certain Leishmania species such as L. b. panamensis in Lu. gomezi, could complete their extrinsic development before the completion of first gonotrophic cycle of female sand flies. Thus, for the transmission of such Leishmania species, females that had completed one gonotrophic cycle should be considered potentially dangerous. Temperature related curves for extrinsic development of Leishmania in sand flies, and the time required for the completion of different gonotrophic cycles during different seasons are not available for most of the sand flies. This lack of information is due to the absence of well established laboratory colonies of sand flies.

Some species were capable of _Leishmania_ transmission during most of the year; other information is also required to draw definite conclusions regarding vector potential. There remains a need for additional studies related to host preferences and genetic susceptibility of sand flies to _Leishmania_ parasites.

CHAPTER 7
SUMMARY AND CONCLUSIONS

The present study was designed to investigate different aspects of the biology of Lutzomyia sand flies such as mating, food and blood meal digestion in immatures and females respectively, and JH production in females. Field populations of eight different Lutzomyia species from Colombia were age graded to determine their longevity and potential role in the transmission of Leishmania.

Females of Lu. anthophora were sexually mature and able to mate few hours after emergence. The maximum number of 1-day-old females mated with sexually mature males. Male emerged earlier than the females and were sexually mature within 13 hrs after emergence. The greatest number of females were inseminated by 24 to 48-hr-old males. Each male was capable of inseminating 1-5 females during the present experimental conditions. Males also mated with blood fed females. Time for a successful mating was much longer than that observed in mosquitoes and ranged from 3 to 38 minutes.

Different organs of the male reproductive system changed significantly due to the effects of age and mating. Newly emerged virgin males had the largest testes, whereas those of 7 day old virgin males were the smallest. Peak spermatogenesis occurred in 0 to 2-day-old males which had the greatest number of mature spermatocysts. Numbers of total and mature spermatocysts were maximum in 0-day-old males and minimum in 7-day-old males. The amount of sperm in the sperm reservoir increased as the males became older. Similarly, more accessory gland substance was secreted in older males. The accessory glands were largest in 2-day-old males and such males inseminated the greatest number of females. The amount of accumulated sperm increased in the seminal vesicles of older males.

The testes became smaller in size due to increasing age and multiple inseminations and smallest testes and accessory glands were present in 5-mated males. No change was observed in the number of spermatocysts due to multiple matings. The size of the sperm reservoir decreased with multiple matings. Four to 5 mated males had the smallest sperm reservoir. Similarly, the accessory glands of virgin males were largest and one mated males had larger accessory glands than 2-5 mated males. Five mated males had the narrowest width of seminal vesicles.

Males that inseminated several females transferred the maximum amount of accessory gland fluid to the first female rather than subsequent partners. However, it is not known whether the male accessory gland fluid renders female sand flies refractory to further inseminations as was shown in mosquitoes. Females were inseminated by more than one male in the colony cages. It is not known if multiple inseminations, within a short time, occurs in nature. Females were ready to take a blood meal 3 days after emergence and eggs were laid 5-6 days after blood feeding (Endris 1982). The eggs hatched after 6 days under the present experimental conditions and larvae molted to pupae after 17 days.

Lutzomyia *anthophora* has four larval instars. The first lasts 5 days, the second and third for 3 days, and the fourth instar larvae molted to pupae after 6 days. Adults emerged after 8 days.

The amount of trypsin- and chymotrypsin-like enzymes produced by immature stages and adult females of *Lu*. *anthophora* was determined. The amount of serine proteases was smallest in first instar larvae but increased significantly in later instars. The maximum amount of serine proteases was produced by the midguts of 12 to 16-day-old larvae. The amount of serine proteases produced by first instar, second instar larvae, pupae, 0 to 2-day-old females, 3-day-old sugar fed females, and females 96 hrs after blood feeding were not significantly different. Similarly, the amount of serine proteases produced by third instar larvae, females 12 to 24-hrs and 30-38-hrs after blood feeding did not differ. No difference was detected in the amount of total serine proteases produced by fourth instar larvae and females 48-hrs after blood feeding.

The amounts (mean ± SEM ng) of trypsin-like enzymes produced by first instar larvae (1.13 ± 0.05 ng), second instar larvae (5.57 ± 0.57 ng), third instar larvae (11.84 ± 0.46 ng), and pupae (7.54 ± 0.95 ng) were not significantly different. The smallest amount (1.13 ± 0.05 ng) of trypsin was secreted by first instar larvae and maximum amount (36.84 ± 5.74 ng) was secreted by fourth instar larvae. No significant difference was present in the quantities (mean ± SEM ng) of chymotrypsin secreted by first instar (3.21 ± 0.53 ng), second instar larvae (18.73 ± 2.89 ng), and pupae (3.38 ± 0.51 ng). Third instar larvae (41.97 ± 3.50 ng) secreted 12.4 times more chymotrypsin than the first instar larvae. Similarly, two fold more chymotrypsin was secreted by fourth instar larvae (92.68 ± 12.54 ng) compared to third instar larvae. The maximum amount (144.19 ng) of chymotrypsin was secreted by the midguts of 16-day-old larvae. More chymotrypsin than trypsin was secreted by 2-day-old sugar fed females (5.06 ng) than those 3-day-old (3.35 ng) and those that took a blood meal 72 hrs earlier (209.19 ng).

Greater amounts of trypsin-like enzymes were secreted after blood feeding compared to chymotrypsin-like enzymes, except at 72 hrs when more chymotrypsin-like enzymes were secreted. Twelve different isozymes were secreted by larvae and 8 of these were trypsin-like. Newly-formed pupae had 8 minor isozymes but only one was chymotrypsin-like. Older pupae had four isozymes, one of which was chymotrypsin-like. Newly-emerged females had 4 trypsin- and chymotrypsin-like isozymes. One-day-old sugar fed females had 5 isozymes but sugar fed females had only three isozymes. Eleven different isozymes were secreted by blood fed females; two of these were purely chymotrypsin-like and 2 had both trypsin- and chymotrypsin-like activities.

Egg development closely followed blood digestion. Primary follicles of newly-emerged females are very small and cannot be differentiated into nurse cells and an oocyte. Following sugar feeding, the primary follicles increased nonsignificantly in size and 3-day-old sugar fed females have resting stage ovarioles. Stage N (or resting stage) follicle had 7 monomorphic nurse cells and an oocyte. Secondary follicle could not be distinguished from the primary follicle at this time.

The primary follicle became significantly larger within the first 24 hrs after blood feeding due to yolk deposition. The secondary follicle could be distinguished from the primary follicle at this time. A significant increase occurred in ovariole size and the amount of yolk inside the follicle within 24-72 hrs. The maximum amount of yolk was present after 96 hrs and the eggs were completly mature at this time. Blood digestion was almost complete after 72 hrs and fourth stage eggs were present in the ovaries. The size of the ovariole, 96 hrs after blood feeding, was similar to the size of the eggs that were deposited 96-120 hrs after blood feeding.

Changes in egg development, when correlated to the amount of JH III produced *in vitro*, showed similar changes as were observed earlier in different mosquitoes. In *Lu. anthophora*, JH III biosynthesis increased in unfed and sugar fed females within the first 24 hrs after emergence. The rate of JH III biosynthesis reached its greatest level in 1-day-old sugar fed females. Later, the rate declined in 3-day-old sugar fed females. Three-day-old sugar fed *Lu. anthophora* had resting stage ovarioles. In mosquitoes, newly emerged females become reproductively competent due to a chain of events that start by the release of JH III from the corpora allata. Juvenile hormone III stimulates the primary follicles of newly emerged females to grow to a previtellogenic stage called the resting stage (Lea 1963; Gwadz & Spielman 1973). In *Ae. aegypti*, JH III level is high during the first two days after emergence but then it gradually declines. Rosignol et al. (1981) and Shapiro et al. (1986) suggested that the decline in JH levels is due to feed back inhibition by resting stage ovaries. The mode of JH III biosynthesis in newly emerged and sugar fed female *Lu. anthophora* was very similar to mosquitoes. The factors that control JH III production in Lu. anthophora are not yet known.

A nonsignificant decline occurred in the rate of JH III biosynthesis just after blood feeding but increased after 2 hrs and was maximum at 4 hrs after feeding. The rate of JH III biosynthesis was similar to that of 1-day-old sugar fed females. The rate of JH III biosynthesis declined later and the minimum amount of JH was synthesized 30 hrs after blood feeding. The rate increased 38 hrs after blood feeding and greatest amount of JH III was synthesized 96 hrs after blood feeding. At this time, the ovarian follicles were completely mature.

The fat bodies of mosquitoes lose their ability to respond to 20-OH-ecdysone 20-hrs after blood feeding (Bohm et al. 1978) and a new burst of JH III is required to produce another egg batch (Hagedorn 1983). It is not known if such a mechanism operates in Lu. anthophora. A new supply of JH III is required after each oviposition in Culex mosquitoes for the initiation of biting behavior (Meola & Petralia 1980; Meola & Readio 1987). On the other hand, JH does not seem to control the biting behavior in Ae. aegypti and its role in the biting behavior of Lutzomyia sand flies is not known.

Females of different Lutzomyia species survived for different periods of time in nature in Colombia. Lutzomyia cayennensis females completed 4 gonotrophic cycles in

August. The maximum number of parous females were collected during January 1988; females that had completed two gonotrophic cycles were found during March and May-August. Based on the trophic status of females from tree trunk resting collections, there is a possibility that fresh fed females rest elsewhere. Females were capable of *Leishmania* transmission during most of the year except January 1987, February, and October. Of course, their vectorial capacity will depend upon all factors such as their population density, anthropophily, presence of other animal hosts in the area and the chances of man vector contact.

Lutzomyia *ovallesi* an anthropophilic species, used the tree trunks as the preferred resting sites. Females that had completed 2 gonotrophic cycles were collected during March, August, and September. Females laid three egg batches during June and September and 15% of females had completed 2-3 gonotrophic cycles in September at Arboledas. Females took multiple blood meals during one gonotrophic cycle in May and January thus, increasing the chances of *Leishmania* transmission.

Lutzomyia *serrana* females were capable of *Leishmania* transmission during January and March when they completed two gonotrophic cycles. Small number of nulliparous unfed females were caught from the tree trunks during April and May 1986. This was a period of peak rainfall. A high mortality of larvae and pupae might have occurred in this season due to the destruction of the larval breeding habitat by heavy rains.

Lutzomyia shannoni was capable of Leishmania transmission throughout the year. Females completed three gonotrophic cycles in February and April at Arboledas. Since the females are autogenous in the first gonotrophic cycle, females that had completed at least two gonotrophic cycles were considered potentially dangerous. Parous females came to bite at dusk, and females that had completed 2 gonotrophic cycles came to bite only in July, August and September. All night human bait collection are required to understand the role played by this species in disease transmission during the rest of the year. Females that had completed 2 gonotrophic cycles were found resting on tree trunks throughout the year and those that had completed 3 gonotrophic cycles were captured in January and April.

Lutzomyia spinicrassa is the suspected vector of Le. braziliensis at the present study sites (Young et al. 1987). Females completed 2 gonotrophic cycles from April-December and were capable of transmission during these months. Parous females were encountered throughout the year. Females took multiple blood meals during one gonotrophic cycle from September to December and thus, increased the chances of Leishmania transmission.

Lutzomyia trinidadensis was one of the longest lived species collected during the present study. Females completed up to 4 gonotrophic cycles in June and September. Potentially dangerous populations of Lu. trinidadensis were found during March, May-September and December but they comprised a small percent of the total population. Although most of the previous studies indicate that it to feeds on reptiles a few nulliparous females were found biting man during May and January.

Small populations of Lu. venezuelensis were present at Durania throughout the year. Females completed three gonotrophic cycles in May and September. Female Lu. erwindonaldoi completed 2-4 gonotrophic cycles in March-August 1987. During August, one female that had oviposited 4 times earlier came to bite man. Due to its long survival it can be considered a potential vector at Arboledas and Durania.

Reproduction in Lutzomyia sand flies is a complex network of different physiological processes, some of which were investigated in the present study. All of the Lutzomyia species investigated in the present study could potentially transmit Leishmania during some season of the year, but there is little information available on transmission seasons at these localities (Alexander 1988). Most of the species were capable of transmission during March-September and a few others were active throughout the year. More information such as blood feeding habits, biting patterns, population density of the sand flies are required to discover the major vector of the disease in the area. The current information suggests that *Lu*. *spinicrassa* may be the sole vector of *Le*. *braziliensis* in the present study site.

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BIOGRAPHICAL SKETCH

Farida Mahmood was born on 7th January 1953 in Faisal Abad, Punjab, Pakistan. She grew up in Lahore, Punjab, and received her high school certificate from the Government Girls High School, Whadat Colony, Lahore, Pakistan. She received her bachelor's degree in science from the Lahore College for Women, Lahore, Pakistan, in 1973. She received her master's in botany in 1976 from the University of Punjab, Lahore, Pakistan, and stood second in the university.

She received a merit scholarship from Fauji Foundation from 1969-1971. Later she was awarded a merit scholarship by the government of Pakistan in 1973. She worked as a field research investigator in the Department of Social Sciences at the University of Punjab in the beginning of 1977. She joined the Ecology Department, Pakistan Medical Research Center, University of Maryland, School of Medicine, Lahore, Pakistan, in September 1977. She received training in mosquito ecology from September 1977 to October 1978.

In 1978 she was selected as a lecturer in biology by the Punjab Public Service Commission and stood second in the examination. She taught biology from October 1978 to

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She enrolled in University of Florida in August 1984 and received her Master of Science degree in August 1986. During this time she was employed as graduate research assistant at Florida Medical Entomology Laboratory, Vero Beach, Florida, USA. She finished her research under the supervision of Professor J. K. Nayar. Her research was on the physiological responses of susceptible and refractory strains of Aedes aegypti L. to Dirofilaria immitis (Leidy) infection.

She enrolled in PhD program in entomology and nematology with an emphasis in medical entomology and worked as a graduate research assistant at University of Florida Gainesville, Florida, from August 1986 to December 1988. She finished part of her research in Professor J. F. Butler's laboratory under the kind supervision of Dr. D. G. Young. In 1989 she moved to Florida Medical Entomology Laboratory, Vero Beach, and finished rest of her research in

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I certify that I have read this study and that in my opinion it conforms to acceptable standards of scholarly presentation and is fully adequate, in scope and quality, as a dissertation for the degree of Doctor of Philosophy.

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